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This issue in two Parts

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This issue of the *Scientific Edition* of the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION is supplied in two parts. Part I is the customary edition of the JOURNAL, and Part II is a supplement carrying the list of active members of the AMERICAN PHARMACEUTICAL ASSOCIATION, the roster of committees and delegates, a brief history and summary of the ASSOCIATION'S activities, the list of officers of the ASSOCIATION and places of meeting since its organization, the Constitution and By-Laws, the Certificate of Incorporation, the Code of Ethics, the lists of national, state and local pharmaceutical organizations with the names of their Secretaries, the list of accredited Colleges of Pharmacy, State Boards of Pharmacy, and other similar information.

Prior to 1945, the PROCEEDINGS of the AMERICAN PHARMACEUTICAL ASSOCIATION were published either separately or in installments in the monthly JOURNAL. Since 1949, we have been issuing the information referred to in the previous paragraph as a supplement to the *Scientific Edition* of the JOURNAL so that it could be available as a separate volume to those who refer to this information frequently. The PROCEEDINGS of the Annual Convention are published in narrative form in the *Practical Pharmacy Edition* of the JOURNAL immediately following the annual convention.

The PROCEEDINGS of the 1951 convention were published in narrative form in the September, 1951, number of the *Practical Pharmacy Edition* of the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION. Later editions of the JOURNAL carried the addresses and reports of special interest. Many of the scientific papers and those dealing with practical pharmacy, economics, history, education, and legislation which were presented at the various section meetings have been or are being published in various issues of either the *Scientific* or *Practical Pharmacy Edition* of the JOURNAL.

In addition, there will be made available to the officers and members of the House of Delegates of the ASSOCIATION, to active members desiring it, and to libraries, a condensed stenographic transcript of the general sessions and the sessions of the House of Delegates at the 1951 convention, omitting the addresses and reports published in the JOURNAL. Copies of these PROCEEDINGS will be available to members of the ASSOCIATION without charge, and to nonmembers at $2.00 per copy.

The membership list, which is a part of the supplement to the February, 1952, *Scientific Edition*, includes active members in good standing as of October 31, 1951. Members who joined the ASSOCIATION after October 1, 1951, have been listed in one of the issues of the *Practical Pharmacy Edition* of the JOURNAL appearing since that date, and their names will, of course, be included in the next supplement to be published early in 1953.

Members of the ASSOCIATION are earnestly requested to check the accuracy of the address given with their names and send us any necessary changes.

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In preparing manuscripts for publication in THIS JOURNAL, center headings should be avoided as far as possible. Side headings followed by a period and dash should be indented and run into the part of the text to which they apply. Such side headings may be used as freely as desired. Literature citations should be numbered consecutively in order of appearance in the manuscript and should be indicated by full-sized Arabic numerals enclosed in parentheses. If a literature reference is cited more than once in a manuscript, subsequent citations should be indicated by the first number assigned. References to footnotes throughout the text should be numbered consecutively by superscript Arabic numerals, but such references in each table should be designated by superscript lower case letters beginning with "a" and continuing in natural order.

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The next annual meeting of the American Pharmaceutical Association will be held in Philadelphia, Pennsylvania, August 17 to 23, 1952. Members planning to present papers before any Section during this meeting are earnestly requested and strongly urged to notify the appropriate Section secretary of their intention promptly. Titles and abstracts (not to exceed 250 words) of papers should be submitted by May 15, 1952, or preferably earlier, in order to insure a place on any Section program. Send titles and abstracts of papers to:

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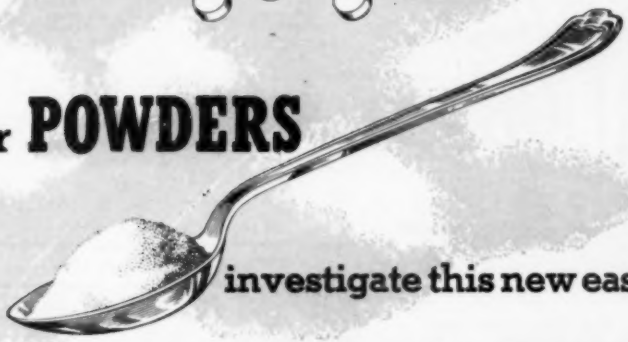

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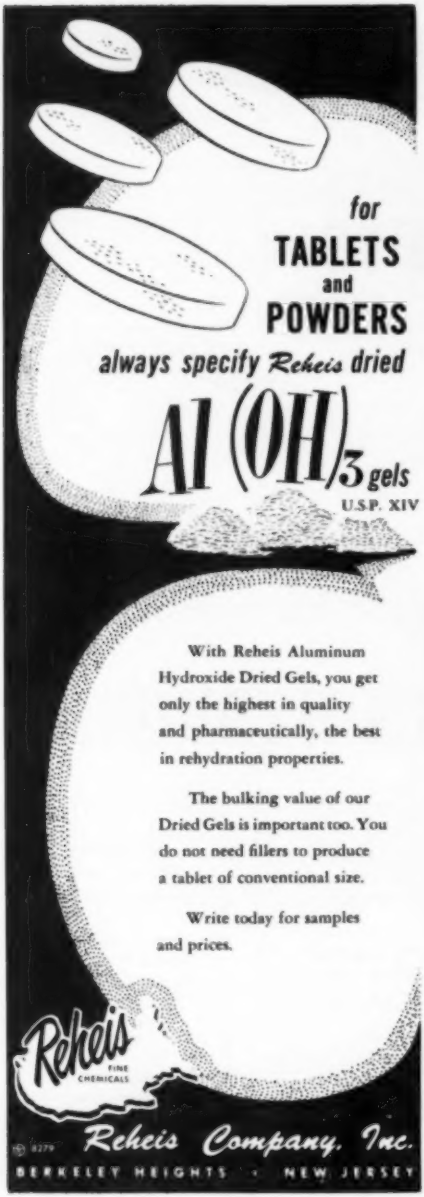
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VOLUME XLI | *FEBRUARY, 1952* | NUMBER 2 | CONSECUTIVE NO. 4

A Study of the Resistance of Skin Micrococci to Hexachlorophene*

By THOMAS C. GRUBB and HOYLAND A. WANDS

Micrococci were isolated from the hands of eight subjects before and after using a lotion containing 0.3 per cent hexachlorophene three times a day for seven weeks. Agar plates were inoculated with the 32 cultures of micrococci isolated, and resistance to the antiseptic was determined by the diameter of the zone of growth inhibition around the agar cups containing the G-11 lotion. No difference in the resistance of the micrococci isolated before and after using the G-11 lotion was detected.

THE USE of hexachlorophene (G-11) in soap and other products for the purpose of "degerming" the skin has become more extensive within the last few years because there seems to be no question of its ability to reduce the microbial flora of the skin, and also there is no indication that it produces any significant irritation or sensitization of the skin when properly used. In the course of determining the degerming action of a cosmetic-type of lotion containing 0.3 per cent of G-11, the opportunity presented itself to ascertain whether or not the skin bacteria, especially the micrococci, develop any resistance to the antiseptic. This problem has not been previously studied, so far as the writers are aware, although the question has been raised (1).

EXPERIMENTAL

A group of 16 subjects (housewives) was employed in this test, half using a lotion containing G-11 on their hands three times a day (after washing the dishes at mealtime), and the other half using the same lotion without G-11 in a similar way. No soap or other products containing G-11 were used by these groups during the experiment. The number of bacteria on the hands was determined by a modified Price technique (2) before any lotions were used and once a week at the same time for the following seven weeks. It is important to note that the hand-washing tests were carried out not less than two hours after the previous application of the lotion and that 2% sterile beef serum was added to all plates (from both groups) to inactivate any possible carry-over of the G-11. As a check to determine whether or not sufficient G-11 had been carried over in the washings to produce significant bacteriostasis, a number of the plates from subjects in the test group that showed very few colonies and uninoculated control plates of nutrient agar were streaked with a very dilute suspension of *Staphylococcus aureus*. After suitable incubation, the number of colonies in the control plates was found to be approximately equal to the number on the subjects' plates, *minus* the number found after the initial incubation. This suggests that an inhibitory amount of G-11 was not present in the subculture plates.

The relative number of bacteria washed from the hands of the subjects in the control and test groups is shown in Fig. 1.

The number of bacteria in the washings from the hands of the test and control group was approximately the same before the lotions were used and a

* Received September 17, 1951, from the Vick Chemical Company, New York, N. Y.

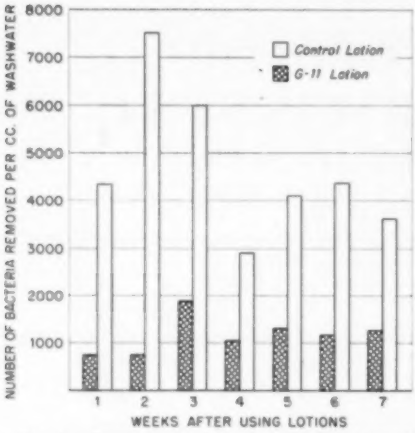

Fig. 1.—Comparison of average bacterial counts from subjects using a lotion with and without G-11.

much smaller number of bacteria was washed from the hands of the G-11 group than the control group each week the lotions were used. There was a 75% reduction in bacterial count of the washings as compared with the control group, for the seven-week test period.

The experiment to determine whether or not the bacteria on the skin of the subjects using the G-11 lotion became resistant to the antiseptic during the seven weeks of daily application was carried out as follows: Colonies were fished from the plates after the first washing of the subjects' hands in the G-11 group before the lotion had been used, and grown on agar slants before storage in the refrigerator. In a similar fashion, colonies were picked from the plates of the last washing after the subjects had used the lotion daily for seven weeks. All slants were then microscopically examined and two pure cultures of micrococci isolated from each subject before the lotion was used and two pure cultures of micrococci after it had been used were selected for final testing. It was decided to study only the micrococci since they are the most numerous on the skin and the degerming action of G-11 is mainly due to its action on the micrococci. It is important to note that only two transfers were made with the strains isolated at the last washing before the test for their resistance to the antiseptic.

The selected cultures were inoculated into nutrient broth and incubated eighteen hours at 37°. Each culture was then diluted with nutrient broth until all were about the same density (approximately 112,400,000 bacteria per cc.). One-tenth cubic centimeter of each culture was placed in a Petri dish and exactly 10 cc. of melted nutrient agar intimately mixed with the inoculum and allowed to solidify. Four cups, one in each quadrant of the plate, were made in the agar with a cork borer 11 mm. in diameter. The bottom of the cups was not sealed with melted agar because it was unnecessary to evaluate the factor of diffusion where the same liquid was being used in all cups. Two-tenths cubic centimeter of the lotion containing 0.3% G-11 was placed in each of the cups and all plates were incubated eighteen hours at 37° when the diameter of the zones of growth inhibition around each of the 128 cups was measured. The average diameter of the zone of inhibition for the cultures taken before G-11 lotion was used was 26.7 mm. with a standard error of ±0.18 mm.; while that of the cultures isolated after using the G-11 lotion was 26.4 mm. with a standard error of ±0.37 mm. This indicates that the micrococci isolated and tested as described above did not develop any resistance to G-11.

DISCUSSION

Although a conclusive answer to the question of whether or not any types of skin bacteria become resistant after repeated applications of G-11 cannot be given until the Gram-positive and negative, aerobic and anaerobic flora are tested, this study suggests that micrococci, the predominating type of aerobic bacteria on the skin (3), do not become resistant under the conditions of this experiment. Whether or not resistant strains would emerge after longer periods of application of the antiseptic has not been directly determined, but it would appear very unlikely since continuous application of G-11 soap for twelve months in one study (4) and eight months in another (5) failed to disclose any upward trend in bacterial counts as the studies continued. This is what one would expect if resistant strains of any of the skin flora failed to develop.

While a variety of methods are available for testing the resistance of a given microorganism to antiseptics, the method selected is believed to be valid, simple, and reproducible. It is well known that Gram-negative bacteria are not as susceptible to G-11 as the Gram-positive organisms. For example, when the cup-plate method described above was used with *Escherichia coli*, a zone of inhibition measuring 12–13 mm. in diameter was obtained, thus indicating that the resistance of the organism is correlated with the size of the inhibition zone.

SUMMARY

The application of a lotion containing 0.3 per cent G-11 three times daily to the hands of eight subjects was found to lower the average total bacterial count of the washings by approximately 75 per cent when compared with a similar group using the same lotion without the antiseptic. No difference in resistance to G-11 was detected between the cultures of micrococci isolated before and after using the lotion containing G-11, as determined by an agar cup test using 32 strains of organisms.

REFERENCES

(1) Report of the Committee on Cosmetics, *J. Am. Med. Assoc.*, **142**, 814(1950).
(2) Price, P. B., *J. Infectious Diseases*, **63**, 301(1938).
(3) Evans, C. A., Smith, W. M., Johnston, E. A., and Giblett, E. R., *J. Investigative Dermatol.*, **15**, 305(1950).
(4) Fuller, J. R., Newhall, C. A., Thorne, F. C., and Taube, E. F., *Am. J. Pub. Health*, **38**, 9(1948).
(5) Blank, I. H., and Coolidge, B. A., *J. Investigative Dermatol.*, **15**, 257(1950).

Synthesis of Diphenic Acid Derivatives*

By FRANCOIS X. DEMERS, Jr.,† and GLENN L. JENKINS

Derivatives of diphenic and diphenamic acids were prepared for pharmacologic evaluation as possible antihistamines or antispasmodics. In the diphenic acid series, the monoethyl diphenates of diethyl- and dimethyl-aminoethanols were prepared, as well as the hydrogen diphenates of these alcohols and of 2-(N′,N′-dimethyl-N-phenylethylenediamino)-ethanol. A series of N-(2-dimethylaminoethyl)-N-R diphenamic acids and ethyl diphenamates, in which R represents phenyl, benzyl and 2-pyridyl, were prepared. In addition to the above, the following new compounds were prepared: ethyl 2-chloroformyl-2′-biphenylcarboxylate, 2-(N′,N′-dimethyl-N-phenylethylenediamino)-ethanol, N-(2-chloroethyl)-N-phenyl-N′,N′-dimethyl-ethylenediamine dihydrochloride, 1,1-dimethyl-4-phenylpiperazinium chloride, ethyl N,N-diethyl diphenamate, and N-(2-hydroxyethyl)-diphenimide.

THE PURPOSE of this investigation was to make available for pharmacologic evaluation, as possible antihistaminics or antispasmodics, a number of substituted amino esters and amides of diphenic acid and of ethyl hydrogen diphenate.

Precedence for antihistaminic activity in esters is furnished by the diethylaminoethyl esters of fluorene-9-, 9-xanthene, and 9,10-dihydroanthracene carboxylic acids reported by Burtner and Cusic (1). During the course of this work, antihistaminic activity was reported (2) for a series of amino esters of substituted alicyclic carboxylic acids, and some of these showed a striking structural similarity to a few of the compounds prepared in this work. At the onset of this investigation, no antihistamine compound having an amide structure was known to the authors. However, in view of the close structural relationship of amides to esters, it seemed logical to anticipate antihistaminic properties in appropriately substituted amides. This view was subsequently confirmed by the appearance (3) of two such amide compounds, 2813 RP and SU 186.

In 1925, the hydrochlorides of diethylaminoethyl diphenate and diethylaminoethyl imidoester of diphenanilide were reported (4) to possess local anesthetic action. More recently (1941), diethylaminoethyl-5,5′-diaminodiphenate was prepared (5) and shown to have marked local anesthetic activity.

The compounds reported in this paper are of two general types: (A) those in which a carboxyl group is retained; and (B) those which do not have a functional carboxyl group. Synthesis of type A (Procedure A) simply involved the reaction of diphenic anhydride with the appropriate alcohols to give acid-esters, or with substituted ethylenediamines to give N-substituted diphenamic acids. Synthesis of the B series (Procedure B) was achieved by reaction of ethyl diphenoyl chloride with the substituted alcohols to give mixed esters, or with secondary amines to give ethyl diphenamates. The mixed esters were prepared (Procedure C) by another method which consisted of reacting potassium ethyl diphenate with a 2-dialkylaminoethyl chloride. These procedures are illustrated under Experimental by specific examples, and the compounds thus prepared are listed in Tables I and II. In the course of this work, a diphenimide derivative was prepared, and in an attempt to prepare a second, a piperazinium compound was obtained.

EXPERIMENTAL[1]

Materials.—Diphenic acid was prepared according to "Organic Syntheses" (6) with the modification that, in the precipitation of the product, the addition was reversed, i.e., the hot diphenate solution was added to the hydrochloric acid. The modification avoided the formation of small lumps of copper compounds. The procedure of Roberts and Johnson (4) for the preparation of diphenic anhydride was followed with the exception that, instead of recrystallizing the product from acetic acid, it was washed with alcohol-free ether.

Ethyl 2 - Chloroformyl - 2′ - biphenylcarboxylate (Ethyl Diphenoyl Chloride).—To 84 Gm. of ethyl hydrogen diphenate (7), dissolved in 225 cc. of dry benzene, was added 33.3 cc. (0.4 mole) of thionyl chloride, and the mixture was refluxed for eight hours on a steam bath. The solvent was removed by distillation, and the product fractionated; b. p. 195° (6.5 mm.); m. p. 47°; yield 95%.

Anal.—Calcd. for $C_{16}H_{13}ClO_5$: Cl, 12.27. Found: Cl, 12.13.

N-(2-Hydroxyethyl)-N-phenyl-N′,N′-Dimethylethylenediamine.—2 - Bromoethyldimethylammonium bromide (8), (270 Gm., 1.16 moles), was dissolved in 625 cc. of hot 99% ethanol, 159 Gm. (1.16 moles) of 2-anilinoethanol (9) was added, followed

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† Fellow of the American Foundation for Pharmaceutical Education. Present address: Detroit Institute of Technology, School of Pharmacy, Detroit, Mich.

[1] Melting points are uncorrected.

by 160 Gm. of anhydrous potassium carbonate, added in approximately five equal portions. The mixture was refluxed and stirred for seven and one-half hours. After filtration, the solid was washed with 100 cc. of ethanol, and the filtrate was evaporated under reduced pressure. The residue was dissolved in ca. 300 cc. of water, 46 Gm. of sodium hydroxide was added, and the mixture was extracted with two 50-cc. portions of benzene, the benzene solutions were combined and washed with 50 cc. of water, and dried over anhydrous potassium carbonate. After removal of the solvent, the residue gave 145.7 Gm. (60.3%) of a viscous, nearly colorless oil which boiled at 141–149° (4 mm.), n_D^{25} 1.5093, n_D^{25} 1.5579.

Dihydrochloride.—To 2.6 Gm. of the amine-alcohol dissolved in 25 cc. of ether was added 10% alcoholic hydrogen chloride until the solution became acidic to litmus. The ether was decanted from the oil which separated, and crystallization was induced by rubbing the oil with fresh ether. Recrystallization from 2-propanol gave white crystals, m. p. 105–106°.

Anal.—Calcd. for $C_{12}H_{22}Cl_2N_2O$: N, 9.96. Found: N, 10.15.

N-Benzyl-N′,N′-dimethylethylenediamine.—This compound has been prepared by a method recently published (10) which differs from the one described here, and it has also been reported in a patent (11). The following procedure was based on a patent (12) for the aminoalkylation of amines. One mole (107 Gm.) of benzylamine was added to 0.4 mole (93.2 Gm.) of 2-bromoethyldimethylammonium bromide placed in a 300-cc. flask to which an air condenser was attached. A vigorous reaction took place at once with considerable evolution of heat. After the initial reaction had subsided, the flask was heated in an oil bath, maintained at 140–150°, for five and one-half hours. The temperature was then raised rapidly until a clear solution was obtained (ca. 180°). When cooled, the solution crystallized to a solid mass which was dissolved in 100 cc. of 99% ethanol and to this solution was added 52 Gm. (equivalent to 0.8 mole) of potassium hydroxide dissolved in 200 cc. of ethanol. The precipitated potassium bromide was filtered, and the filtrate was evaporated under reduced pressure. Fractionation of the residual oil gave a nearly colorless oil,[2,3] b. p. 126–132° (14 mm.), d_{25}^{25} 0.9345, n_D^{25} 1.5093, R (calcd.) 56.936, R (exp.) 57.018. The following constants have been reported: (10) b. p. 115–117° at 4 mm., n_D^{25} 1.5052; (11) b. p. 128–132° at 18 mm. The dihydrochloride, recrystallized from absolute ethanol, melted at 205–207° which is the value reported in the literature (10).

Anal.—Calcd. for $C_{11}H_{20}Cl_2N_2$: N, 11.15. Found: N, 10.94.

N-(2-Chloroethyl)-N-phenyl-N′,N′-dimethylethylenediamine Dihydrochloride.—Dry hydrogen chloride was passed into 41.6 Gm. (0.2 mole) of 2-(N′,N′-dimethyl-N-phenylethylenediamino)-ethanol, dissolved in 175 cc. of dry chloroform, until the theoretical quantity (7.2 Gm.) was taken up. To the ice-cold, stirred solution was added dropwise 26.2 Gm. (0.22 mole) of thionyl chloride mixed with 30 cc. of chloroform. The addition was made over a thirty-minute period. The mixture was allowed to warm to room temperature overnight, then the solvent was removed under reduced pressure. Two portions of 20 cc. of benzene were added and stripped off to remove the excess thionyl chloride, and the residue was dried under vacuum over sodium hydroxide for twenty-four hours. A portion was crystallized from acetone, but it was found that the product can be satisfactorily purified by merely washing it with hot acetone. A yield of 54.1 Gm. (90.2%) of a white crystalline powder, which melted at 115–119° (dec.) was obtained.

Anal.—Calcd. for $C_{12}H_{21}Cl_2N_2$: N, 8.93. Found: N, 8.98.

1,1-Dimethyl-4-phenylpiperazinium Chloride.—In an attempt to prepare N,N-dimethyl-N′-phenyl-N′-(2-diphenimidoethyl)ethylenediamine, the chloro base, N-(2-chloroethyl)-N-phenyl-N′,N′-dimethylethylenediamine, which was liberated from its dihydrochloride by the method of Tilford, *et al.* (14), was reacted with potassium diphenimide [prepared by a method described (15) for the sodium compound] in a toluene medium. A white crystalline product, which melted at 229–231° (dec.), was isolated. This substance, however, when boiled for four hours with concentrated hydrochloric acid, failed to yield diphenic acid as would be expected from the hydrolysis of a diphenimide derivative. Hence, it was concluded that the product was a piperazinium compound which was formed by an intramolecular condensation of the chloro base.

Anal.—Calcd. for $C_{12}H_{19}ClN_2$: N, 12.35; Cl, 15.63. Found: N, 12.21; Cl, 15.40.

N-(2-Hydroxyethyl)-diphenimide.—To 7 Gm. (0.0268 mole) of potassium diphenimide, dissolved in 50 cc. of absolute ethanol, was added 2.16 Gm. (0.0268 mole) of ethylene chlorohydrin, and the solution was heated on a steam bath for twenty minutes. The potassium chloride precipitate was filtered and washed with 5 cc. of ethanol. The filtrate was evaporated to the original volume and allowed to cool. Water was added, in small portions, until a permanent turbidity was produced, and the solution was allowed to stand in a desiccator overnight. The crystals which separated were washed with water and air-dried; yield 1.2 Gm.; m. p. 93°.

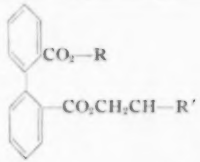
Anal.—Calcd. for $C_{16}H_{13}NO_3$: N, 5.23. Found: N, 5.09.

Procedure A: 2-Dimethylaminoethyl Hydrogen Diphenate Hydrochloride (II, Table I).—A solution of 13.3 Gm. (0.15 mole) of 2-dimethylaminoethanol in 50 cc. of dry benzene was added to 33.6 Gm. (0.15 mole) of diphenic anhydride and the mixture was refluxed on a steam bath until complete solution of the anhydride was effected. The product, which crystallized when cooled, was filtered and crystallized from 2-propanol; yield 33.2 Gm. (70.8%); m. p. 168–169°. The hydrochloride was prepared by adding an ethanolic solution of hydrogen chloride to 15.1 Gm. (0.048 mole) of the monoester dissolved in a minimum amount of absolute ethanol until the solution was acid to litmus. The crystalline product which formed overnight was washed with absolute ethanol, dried in a vacuum desiccator for about

[2] During the evaporation, an estimated 185 cc. of the solution was accidentally lost. Assuming no loss had occurred the yield would have amounted to 36.3% (crude product).

[3] The amine was also obtained in a 19.92% yield by a method (13) used for the preparation of the corresponding phenyl compound.

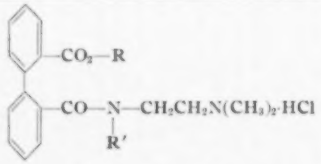
TABLE I.—DERIVATIVES OF DIPHENIC ACID



No.	R	R′	Formula	M. P., °C.	Recryst. Solvent	Yield, %	Procedure	React. Medium	Nitrogen[a] Calcd.	Nitrogen[a] Found	Chlorine[b] Calcd.	Chlorine[b] Found
I	H—	$(CH_3)_2N$—	$C_{16}H_{19}NO_4$[c]	168–169	*i*-PrOH	70.8	A	C_6H_6	4.47	4.26		
II	H—	$(CH_3)_2N$—	$C_{18}H_{20}ClNO_4$	197–198	Abs. EtOH	83.4[d]	..	...	4.00	4.02		
III	C_2H_5—	$(CH_3)_2N$—	$C_{20}H_{24}ClNO_4$	109–111	EtOAc-Et_2O	76.3	B	C_6H_6	3.70	3.37	9.30	9.23
IV	C_2H_5—	$(C_2H_5)_2N$—	$C_{22}H_{28}ClNO_4$	85–86	EtOAc-Et_2O	Poor	C	EtOH	3.45	3.64	8.73	8.90
V	H—	$(CH_3)_2N—C_2H_4—N(C_6H_5)$—	$C_{26}H_{30}Cl_2N_2O_4$[e]	182–186	pet. Et[f]	35.6	A	C_6H_6	5.54	5.38	14.03	14.24

[a] Nitrogen determined by a semi-micro Kjeldahl method; [b] Chlorine determined by the method of Blicke and Zienty (18); [c] Free ester-base; [d] Based on the acid-ester. Over-all yield, 58.7%; [e] Dihydrochloride; [f] This compound was washed with hot ethanol in order to purify it.

TABLE II.—DERIVATIVES OF DIPHENAMIC ACID



No.	R	R′	Formula	M. P., °C.	Recryst. Solvent	Yield, %	Procedure	React. Medium	Nitrogen Calcd.	Nitrogen Found	Chlorine Calcd.	Chlorine Found
I	C_2H_5—	C_6H_5—	$C_{28}H_{29}ClN_2O_7$	139–141	*i*-PrOH-Et_2O	82.9	B	$CHCl_3$	6.18	6.01	7.82	7.73
II	H—	C_6H_5—	$C_{24}H_{25}ClN_2O_5$[a]	127–128	EtOH[b]	36.8	A	EtOAc	6.54	6.40	8.34	8.13
III	C_2H_5—	$C_6H_5CH_2$—	$C_{27}H_{31}ClN_2O_5$	142–144	EtOH[b]	49.3	B	C_6H_6	6.00	6.07	7.59	7.54
IV	H—	$C_6H_5CH_2$—	$C_{25}H_{27}ClN_2O_5$	172–174	EtOH-Et_2O	26.0	A	C_6H_6	6.38	6.20	8.07	7.96
V	C_2H_5—	C_5H_4N[c]	$C_{23}H_{29}Cl_2N_3O_5$	119–121	EtOAc	36.5	B	C_6H_6	8.56	8.58		
VI	H—	C_5H_4N[c]	$C_{21}H_{21}Cl_2N_3O_5$	213–214	EtOH[b]	39.8	A	C_6H_6	9.08	9.22		
VII	Ethyl N,N-diethyldiphenamate		$C_{20}H_{23}NO_3$	72–74	Et_2O-pet. Et	31.7	B	C_6H_6-Pyridine	4.30	4.09		

[a] Picrate, m. p. 214–215°; [b] Absolute ethanol; [c] 2-Pyridyl.

twelve hours, and finally in an oven at 100° for thirty minutes. The product, after recrystallization from ethanol, weighed 14 Gm. (83.4%) and melted at 197–198°.

Procedure B: Ethyl N-(2-Dimethylaminoethyl)-diphenanilate Hydrochloride (I, Table II).—[N,N-Dimethyl-N′-phenylethylenediamine (13, 16) and N,N-dimethyl-N′-(2-pyridyl)-ethylenediamine (16) were prepared by previously published methods.] A solution of N,N-dimethyl-N′-phenylethylenediamine (19 Gm., 0.115 mole) in 30 cc. of dry chloroform was treated with a solution of ethyl diphenoyl chloride (33.1 Gm., 0.115 mole) in 30 cc. of the same solvent. The addition was made in small portions with shaking and occasional cooling in ice water, and then the solution was allowed to stand at room temperature for one hour. Dry ether (about 100 cc.) was then added until a small persistent precipitate was formed, and the mixture was left in an ice bath overnight. The product was filtered, washed with 40 cc. of ether and then dried in a desiccator under vacuum. The product was dissolved in 100 cc. of hot isopropyl alcohol, the solution cooled, and the solution was set in a cool place to crystallize. In order to facilitate the transfer of the bulky solid onto a Büchner funnel, 100 cc. of a 1:1 isopropanol-ether mixture was added, then the compound was washed with 50 cc. of the solvent mixture and finally with 50 cc. of dry ether. The compound was dried under vacuum in a desiccator for six hours, and then in the oven at 100° for one hour. The product thus obtained was a white, bulky, granular powder, which weighed 43.2 Gm. (82.9%) and melted at 139–141° after recrystallization from ethanol.

Procedure C: Ethyl 2-Diethylaminoethyl Diphenate Hydrochloride (IV, Table I).—A solution of 30.8 Gm. (0.1 mole) of potassium ethyl diphenate in 100 cc. of absolute ethanol was prepared as follows: To a solution of 27 Gm. (0.1 mole) of ethyl hydrogen diphenate in 100 cc. of absolute ethanol was added 69 Gm. (0.05 mole) of anhydrous potassium carbonate and the mixture was refluxed on a steam bath until complete solution was effected. To this solution was added 13.5 Gm. (0.1 mole) of 2-diethylaminoethyl chloride (17) and the mixture was refluxed on a steam bath for three hours. The solid, potassium chloride, was filtered and the filtrate was evaporated by drawing a fine current of air through a calcium chloride tube over the solution maintained at 50–60°. Removal of the alcohol caused the separation of more potassium chloride, which was removed by dissolving the ester in ether and filtering. After evaporation of the ether, the residual ester-base was distilled under vacuum (mercury pump). A yield of 20.5 Gm. (55.4%) of a thick yellowish oil, boiling at 205–210° (ca. 0.5 mm.) was obtained. About three-fourths of the distilled base was dissolved in dry ether and, upon passing dry hydrogen chloride into the solution, the hydrochloride separated as a gum. The ether layer was decanted and the product was rubbed repeatedly with fresh portions of ether until it hardened. The product was crystallized by dissolving it in an ethyl acetate ether mixture, adding sufficient ligroin to produce a permanent turbidity, and allowing the mixture to stand. The product melted at 85–86° and was extremely hygroscopic.

The corresponding dimethylaminoethyl compound (III, Table I), was also prepared by this procedure with the exception that the ester-base was not distilled. The product was equally difficult to crystallize and the yield was equally unsatisfactory.

SUMMARY

1. A number of diphenic and diphenamic acid derivatives were prepared for pharmacologic evaluation as possible antihistaminics or antispasmodics.

2. The diphenic acid derivatives include the following:

2-Dimethylaminoethyl hydrogen diphenate and its hydrochloride.
Ethyl 2-dimethylaminoethyl diphenate hydrochloride.
Ethyl 2-diethylaminoethyl diphenate hydrochloride.
2-(N′,N′-Dimethyl-N-phenylethylenediamino)-ethyl hydrogen diphenate dihydrochloride.

3. The diphenamic acid derivatives are as follows:

Ethyl N - (2 - dimethylaminoethyl)-diphenanilate hydrochloride.
N - (2 - Dimethylaminoethyl) - diphenanilic acid hydrochloride.
Ethyl N - benzyl - N - (2 - dimethylaminoethyl) - diphenamate hydrochloride.
N - Benzyl - N - (2 - dimethylaminoethyl) - diphenamic acid hydrochloride.
Ethyl N - (2 - dimethylaminoethyl)-N-(2 - pyridyl) - diphenamate dihydrochloride.
N - (2 - Dimethylaminoethyl) - N - (2-pyridyl) - diphenamic acid dihydrochloride.
Ethyl N,N-diethyldiphenamate.

4. In addition to the above, the following new compounds are reported:

Ethyl 2-chloroformyl-2′-biphenylcarboxylate.
2 - (N′,N′ - Dimethyl - N - phenylethylenediamino)-ethanol.
N - (2 - Chloroethyl) - N - phenyl - N′, N′-dimethylethylenediamine dihydrochloride.
1,1 - Dimethyl - 4 - phenylpiperazinium chloride.
N-(2-Hydroxyethyl)-diphenimide.

REFERENCES

(1) Burtner, R. P., and Cusic, J. W., *J. Am. Chem. Soc.*, **65**, 262(1943); *ibid.*, **65**, 1582(1943).
(2) Tilford, C. H., Van Campen, M. G., and Shelton, R. S., *ibid.*, **69**, 2902(1947).
(3) Scholz, C. R., Abstr. First Nat. Med. Chem. Symposium of the Am. Chem. Soc., Ann Arbor, Mich., June, 1948, pp. 10, 11.
(4) Roberts, R. C., and Johnson, T. B., *J. Am. Chem. Soc.*, **47**, 1396(1925).
(5) Case, H. F., and Koft, E., *ibid.*, **63**, 508(1941).
(6) Atkinson, E. R., and Lawler, H. J., "Organic Syntheses," Coll. Vol. I, John Wiley & Sons, Inc., New York, 1941, p. 222.
(7) Underwood, H. W., and Kochmann, E. L., *J. Am. Chem. Soc.*, **46**, 2072(1924).
(8) Cortese, F., "Organic Syntheses," Coll. Vol. II, John Wiley & Sons, Inc., New York, 1943, p. 91.
(9) Knorr, L., *Ber.*, **22**, 2092(1889).
(10) Gardner, J. H., and Stevens, J. R., *J. Am. Chem. Soc.*, **71**, 1859(1949).
(11) Brit. pat. 606,181, August 10, 1948; through *Chem. Abstr.*, **43**, 3472e(1949).
(12) Schulemann, W., Schönhöfer, F., and Wingler, A., U. S. pat. 1,752,617, April 1, 1930.
(13) Leonard, F., and Solmssen, U. V., *J. Am. Chem. Soc.*, **70**, 2064(1948).
(14) Tilford, C. H., Shelton, R. S., and Van Campen, M. G., Jr., *ibid.*, **70**, 4001(1948).
(15) Wegerhoff, P., *Ann.*, **252**, 18(1889).
(16) Huttrer, C. P., Djerassi, C., Bevars, W. L., Mayer, R. L., and Scholz, C. R., *J. Am. Chem. Soc.*, **68**, 2001(1946).
(17) Slotta, K. H., and Behnisch, R., *Ber.*, **68**, 758(1935).
(18) Blicke, F. F., and Zienty, F. B., *J. Am. Chem. Soc.*, **61**, 776(1939).

The Central Depressive Effect of Carbrital (Pentobarbital and Carbromal)*

By GRAHAM CHEN and CHARLES R. ENSOR

The central nervous depressive effects of pentobarbital sodium and carbromal, both individually and jointly, were determined by their anti-Metrazol activity in rats. At two times their minimal hypnotic dose levels, a maximal anti-Metrazol effect was reached with pentobarbital about fifteen minutes after ingestion; about one hour with carbromal. The duration of effect was five hours for pentobarbital; approximately ten hours for carbromal. Carbrital (1.5 parts of pentobarbital plus 4 parts of carbromal) was shown to produce a rapid and prolonged central depression. The early hypnotic effect is primarily due to pentobarbital while the prolonged sedation is maintained by carbromal. The two also produce their sedative effect synergistically.

CARBRITAL[1] is an oral hypnotic and sedative preparation consisting of pentobarbital sodium, 1.5 gr., and carbromal, 4 gr. in Kapseals,[1] or 2 and 6 gr. respectively in each fluidounce of elixir. It is employed for producing rapid hypnosis and prolonged sedation. The rationale is based upon the laboratory and clinical observations that pentobarbital sodium is essentially hypnotic and sedative, while carbromal is primarily sedative and mildly hypnotic in its central effects.

Recently, a method has been devised by us for the quantitative titration of central depression (1). The anticonvulsant activity of a compound is taken as a measure of its central depressive effect. By such a procedure, the central nervous effects of pentobarbital sodium and carbromal, both individually and jointly, were investigated. The study includes their sedative and hypnotic potencies, the rate of onset, and the duration of action. The results are given in this report.

* Received July 11, 1951, from the Research Laboratories, Parke, Davis and Co., Detroit, Mich.
[1] Parke, Davis and Company registered trade mark.

MATERIALS AND METHODS

Female Sprague-Dawley rats weighing between 100 and 130 Gm., without food for sixteen hours, were used. Pentobarbital sodium and carbromal suspended in 7% gum acacia were administered orally in a volume of 10 ml./Kg. One-half hour later a pentamethylene tetrazol (Metrazol) solution (the convulsant) was injected intramuscularly. The animals were then observed for thirty minutes for convulsions. Three groups of ten rats each were given a dose of the depressant and three graded doses of Metrazol. The doses of Metrazol were so adjusted as to produce convulsions in 20 to 80% of the animals. From the probit log-dose regression line, the CD_{50} (convulsive dose for 50% of the animals) was interpolated (2).

RESULTS AND DISCUSSION

The data obtained in experiments with pentobarbital sodium and carbromal alone are presented graphically in Fig. 1 in which the CD_{50} values of Metrazol and the corresponding doses of the depressant are plotted on the ordinate and on the abscissa, respectively. The three lines for pentobarbital sodium represent with increasing dosage the stage of sedation, hypnosis, and general anesthesia. The intersects of the lines give on the abscissa the minimal hypnotic and anesthetic doses. The slope of

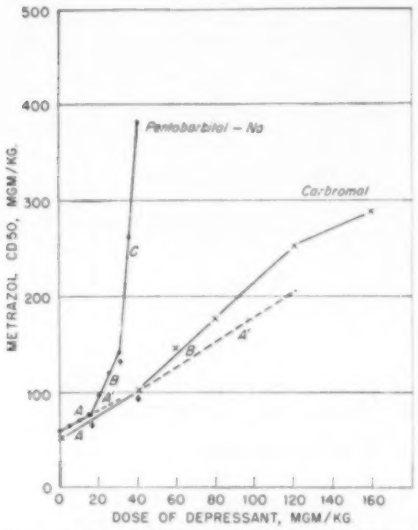


Fig. 1.—The anti-Metrazol effects of pentobarbital and carbromal.

The intersects of the lines shown by arrows give on the abscissa the minimal hypnotic and anesthetic doses. Slope of line *A* represents the sedative potency of each drug. The distance on the ordinate between solid line *B* and the dotted line *A'* shows the depth of hypnosis.

the first lower line signifies a sedative potency while the ordinate at the minimal hypnotic level represents the extent of prehypnotic sedation. The difference in values on the ordinate between the second solid line *B* and the dotted line *A'* shows the depth of hypnosis and this divided by the amount of the depressant required above the minimal hypnotic dose in producing such a stage of hypnosis gives the hypnotic potency. For example, the slope of line *A* for pentobarbital is the ratio of 17 mg./Kg. of Metrazol on the ordinate to 15 mg./Kg. of pentobarbital on the abscissa, a value of 1.2, which indicates the sedative potency of pentobarbital. The difference on the ordinates between the solid line *B* and the dotted line *A'* at 30 mg./Kg. of pentobarbital is 48 mg./Kg. of Metrazol, and this divided by the difference between 30 mg./Kg. and its minimal hypnotic dose (15 mg./Kg.), *viz.*, a ratio of 48/15 or 3.2, is taken as its hypnotic potency. The sedative and hypnotic potencies of pentobarbital and carbromal so calculated, expressed in terms of their anticonvulsant activities, are thus 1.2 and 3.2 mg. of Metrazol per mg. of pentobarbital, 1.3 and 0.6 mg. of Metrazol per mg. of carbromal. The minimal hypnotic doses for pentobarbital and carbromal are 15 and 40 mg./Kg., respectively. The maximal prehypnotic sedation is shown by their anticonvulsant action (at their minimal hypnotic dose levels) equivalent to 18 mg./Kg. of Metrazol for pentobarbital and 70 mg./Kg. for carbromal. As calculated from the slopes of the lines for hypnosis, pentobarbital is about five times more potent than carbromal as a hypnotic agent. Although their sedative potencies are nearly equal, a greater degree of sedation may be induced with larger doses of carbromal than with pentobarbital without producing hypnosis. General anesthesia occurs with 30 mg./Kg. of pentobarbital. It was not possible to produce general anesthesia with carbromal at 2.0 Gm./Kg., a minimal lethal dose.

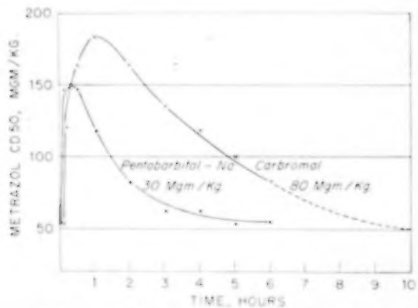


Fig. 2.—The onset and duration of central nervous depression induced by pentobarbital-Na and carbromal as indicated by their anti-Metrazol activity.

In equivalent hypnotic doses, the effect of pentobarbital is quicker, while that of carbromal is more prolonged.

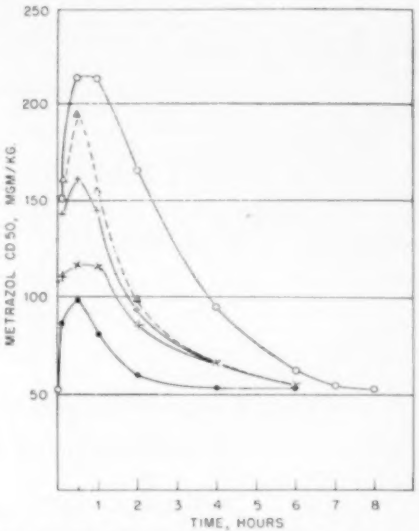


Fig. 3.—The joint central depressive effect of pentobarbital and carbromal (Carbrital).

(1) ● Pentobarbital alone; (2) x carbromal alone; (3) O pentobarbital plus carbromal; (4) + summation of (1) and (2) as experimentally determined; (5) △ calculated from the values on the individual curves for an additive effect of pentobarbital and carbromal, including a joint hypnotic action (see reference 3 for details in calculation).

The rate of onset and the duration of central depressive action of pentobarbital and carbromal are shown in Figs. 2 and 3. In Fig. 2, two times their

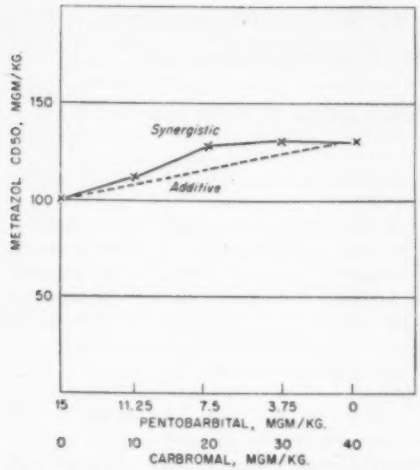


Fig. 4.—The synergistic effect of pentobarbital and carbromal.

The synergistic effect is shown by the experimental curve; an additive effect is indicated by the dotted straight line.

minimal hypnotic doses were used. It is seen that a maximal anti-Metrazol effect was reached with pentobarbital about fifteen minutes after ingestion; about one hour with carbromal. Since carbromal is given in a suspension, the difference in onset may be due to a difference in the rate of absorption of the two drugs from the gastrointestinal tract. After having reached a maximum, the effect of carbromal receded at a much slower rate than that of pentobarbital. The duration of effect was five hours for pentobarbital; approximately ten hours for carbromal.

Similar results are shown in Fig. 3, obtained from an experiment in which the joint effect of pentobarbital and carbromal was also determined with one minimal hypnotic dose of each. Their joint anti-Metrazol effect is seen to be greater than that due to a summation of their individual effects, either by adding the values on the individual curves or as calculated from these values for an additive effect of pentobarbital and carbromal, including a joint hypnotic action (3). This is evident at the fourth hour after ingestion when the effect of pentobarbital had vanished when it was given alone. At that time the effect of the combination was approximately three times greater than the individual effect of carbromal. The enhanced anti-Metrazol activity of the combination may be explained by a synergism of the two in central nervous depression.

The synergistic effect of pentobarbital and carbromal is further shown in Fig. 4 by data which were obtained with equivalent sedative doses of the combination of the two in various proportions. The experimental curve shows their synergistic effect; its curvature represents the intensity of synergism (4). The dotted straight line is for an additive joint action.

CONCLUSION

As determined by the anti-Metrazol activity in rats, Carbrital (1.5 parts of pentobarbital plus 4 parts of carbromal) was shown to produce a rapid and prolonged central depression. The early hypnotic effect is primarily due to pentobarbital, while the prolonged sedation is maintained by carbromal. The two also produce their sedative effect synergistically.

REFERENCES

(1) Chen, G., and Portman, R., "The Determination of Central Nervous Depression," to be published.
(2) Miller, L. C., and Tainter, M. L., *Proc. Soc. Exptl. Biol. Med.*, **57**, 261(1944).
(3) Chen, G., and Portman, R., "The Joint Effect of Central Nervous Depressants," to be published.
(4) Loewe, S., and Muischnek, H., *Arch. exptl. Path. Pharmakol.*, **114**, 313(1926).

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal, and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

3-Methylquinoline
4,4′-Dipiperidine
2,6-Dinitrotoluene
1,8-Dichloronaphthalene
Uvitonic acid
Triisopropyl phosphate
1,3,6-Trihydroxynaphthalene
Thymine-2-desoxyriboside
2-Methyloxazole
Phenyldihydroquinazoline
3-Iodothyronine
3,5′-Diiodothyronine
para-Hydroxyphenyl serine
2-Hydroxynaphthalene-8-sulfonic acid
Ferrous albuminate
Beryllium phthalocyanine
Avidin
Coprostanone
3-Ethyl-2-aminophenylbenzthiazoline
1,4-Dihydroxy-5,8-dichloroanthraquinone

Aminoacyl Derivatives of 2-Aminofluorene and 2,2′-Diamino-9,9′-Spirobifluorene*

By EDWARD V. SVEDRES† and GLENN L. JENKINS

The synthesis of eight new aminoacyl derivatives of 2-aminofluorene and four new aminoacyl derivatives of 2,2′-diamino-9,9′-spirobifluorene are reported. The results of preliminary pharmacological testing of a few representative samples of these new compounds are also reported. A summary is given of negative results in the attempted preparation of analogous aminoalkyl derivatives.

THE FLUORENE NUCLEUS, substituted in number two position, has been utilized in the preparation of a number of compounds which have shown pharmacological activity. Ray and MacGregor (1) prepared a series of 2-(γ-dialkylaminopropyl)fluorene compounds and found them to possess some local anesthetic action. Cusic (2) prepared derivatives of fluorene substituted in the two position with aminoalkyl radicals bearing a substituted oxygen atom. He claimed these compounds to have antispasmodic, antihistaminic, and anesthetic actions. Sargent and Small (3) made a series of compounds which had 2-dialkylamino-1-hydroxyethyl radicals substituted in the two position of fluorene. These were tested and found to possess plasmodicidal activity.

In 1950, Dahlbom and Ekstrand (4) reported the preparation of a series of aminoacyl derivatives of 9-aminofluorene which possessed a high local anesthetic action on the rabbit cornea and weak antihistaminic and antispasmodic actions on the isolated guinea-pig ileum. No aminoacyl derivatives of 2-aminofluorene have been reported in the literature. It was deemed of interest to prepare such a series which could be tested for pharmacological activity. In the present investigation such a series has been prepared and there have been prepared some aminoacyl derivatives of 2,2′-diamino-9,9′-spirobifluorene whose synthesis was reported recently in the literature (5). The methods are reported in the experimental section of this paper.

Numerous attempts were made to prepare the analogous aminoalkyl derivatives of 2-amino fluorene, 2,7-diaminofluorene, and 2,2′-diamino-9,9′-spirobifluorene. All results were mainly negative.

Direct refluxing of ω-(dialkylamino)alkyl halides with 2-aminofluorene produced polymers. Subsequent attempts at condensation were made in solutions of anhydrous benzene, toluene, and xylene under reflux conditions. Small quantities of 2-[ω-(dialkylamino)alkylamino]fluorene derivatives were occasionally obtained in poor yield; however, the results were not reproducible.

The use of mild condensing agents such as sodium bicarbonate did not enhance the attempted condensations. There was evidence that intermediates containing an amino and a halide group cyclized during certain reactions; but, since these products were not of interest, work on them was not pursued.

Attempts to condense substances such as 3-phenoxypropyl bromide with 2-aminofluorene with intent to split the ether linkage with hydrobromic acid and condense dialkylamines with the resultant terminal alkyl bromide were negative.

Various attempts were made to condense aminoalkyl halides with 2,7-diaminofluorene by use of the methods mentioned above. All attempts were negative, yielding polymers and low nitrogen fractions.

Attempts were also made to prepare haloacyl derivatives of 2,7-diaminofluorene as intermediates for an aminoacyl series similar to that of the 2-aminofluorene reported. The use of methods analogous to that of preparing the chloroacyl derivatives of 2-aminofluorene gave insoluble, high-melting, and polysubstituted residues of variable composition.

Other attempts were made to prepare aminoalkyl derivatives of 2,2′-diamino-9,9′-spirobifluorene. All attempts at direct condensation in various organic solvents and using various condensing agents under reflux conditions gave negative results. The use of sodium amide as a condensing agent gave fractions which upon analysis gave the calculated amounts of nitrogen in the theoretical products. These fractions were, however, all amorphous and resinous in character. 2,2′-Bis(diethylaminoacetamido)-9,9′-spirobifluorene, reported in this paper, was treated with lithium aluminum hydride in an attempt to reduce the amido nitrogens to secondary

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† Fellow of the American Foundation for Pharmaceutical Education. Present address: Smith, Kline & French Laboratories, Philadelphia.

amines. The resultant product was amorphous and resinous similar to the products of the direct condensations mentioned above, where sodium amide was used. The use of other common methods reported in the literature for alkylating aromatic amines gave negative results when applied to alkylating the amino groups of 2,2′-diamino-9,9′-spirobifluorene with aminoalkyl substitutions.

The method described in this paper for acylating the spiro diamine with chloroacetyl chloride was similarly applied in an attempt to prepare the analogous β-chloropropionyl derivative as an intermediate for the preparation of an analogous β-aminopropionyl series. β-Chloropropionyl chloride reacted with the spiro diamine to form an amorphous residue of indeterminate composition.

A few representative samples of both aminoacyl series prepared were specifically tested for antispasmodic and local anesthetic activity. All preliminary tests indicated sufficient antispasmodic action to warrant more extensive testing of the entire series. Tests for local anesthetic action on a rabbit cornea gave little or no activity. Intradermal injection of rats, however, indicated some local anesthetic activity. It should be noted that for these tests hydrochloride solutions were prepared by dissolving the compounds in the appropriate amount of a dilute hydrochloric acid solution. It was found that these hydrochlorides were only slightly soluble. This low solubility limits the absorption and thus the pharmacological action of these compounds.

EXPERIMENTAL[1]

2-Aminofluorene.—This material was prepared by the procedure of Kuhn (6) as modified by Schulman (7). A yield of 76% was obtained for the nitration and a yield of 90% was obtained in the reduction.

2-(Chloroacetamido)fluorene.—This compound was prepared by use of the method of Dahlbom and Ekstrand (4) for the preparation of the analogous 9-(chloroacetamido)fluorene. 2-Aminofluorene successfully condensed with chloroacetyl chloride and the product obtained was recrystallized from ethanol and gave a 79% yield of fine pale yellow needles melting at 190–193°. Feldman (8) had reported this compound melting at 183–185°.

Anal.—Calcd. for $C_{15}H_{12}ClNO$: N, 5.43. Found: N, 5.44.

2-(β-Chloropropionylamino)fluorene.—The procedure used was that of Dahlbom and Ekstrand (4) for preparing the 9-aminofluorene analog. Condensation of 2-aminofluorene and β-chloropropionyl chloride gave a yield of 86.5% of crude product. Recrystallization from ethanol gave a 73.5% yield of white micro-plates melting at 189°.

Anal.—Calcd. for $C_{16}H_{14}ClNO$: N, 5.15. Found: N, 5.12.

2-(Diethylaminoacetamido)fluorene.—Five grams (0.019 mole) of 2-(chloroacetamido)fluorene and 4.0 Gm. (0.055 mole) of diethylamine were refluxed in 100 cc. of anhydrous toluene for fourteen hours. The reaction mixture was cooled and extracted with 1 *N* hydrochloric acid. The extract was filtered and upon neutralization with dilute sodium hydroxide solution yielded 5.0 Gm. of white powder. Recrystallization from a methanol-water mixture yielded 4.6 Gm. (81%) of fine pale yellow needles melting at 125–126°.

Anal.—Calcd. for $C_{19}H_{22}NO$: N, 9.50. Found: N, 9.59.

Table I contains the aminoacyl compounds which were prepared by the procedure described above for the compound 2-(diethylaminoacetamido)fluorene. The various dialkylamines and nitrogenous heterocycles were successively condensed with the haloacyl derivatives of 2-aminofluorene. Several condensation reactions were run for the various compounds. In general it was found that two to four equimolar amounts of dialkylamine or nitrogenous heterocycle were required to effect best yields. Reaction times varied from fourteen to thirty-five hours, the criterion being the amount of insoluble amine hydrochloride formed in the reaction mixture. Anhydrous toluene served as a suitable reaction solvent; it was found that about 100 cc./5 Gm. of haloacyl derivative was necessary to effect initial solution in the reaction mixture. The various recrystallization solvents used are listed in Table I.

2,2′-Diamino-9,9′-spirobifluorene.—This compound was prepared with 2-iodobiphenyl and fluorenone as starting materials, these in turn having been prepared by known methods. The preparation procedure was based on the work of Weisburger, *et al.* (5). It was found that the step involving the nitration of 9,9′-spirobifluorene required special insulation of the reaction flask and a very vigorous reflux of the reaction mixture for four hours. Failure to do so gave incomplete nitration.

2,2′-Bis(chloroacetamido)-9,9′-spirobifluorene.—Twenty grams (0.047 mole) of crystalline 2,2′-diamino-9,9′-spirobifluorene, containing one mole of benzene of crystallization, was dissolved in 200 cc. of anhydrous benzene. This solution was diluted with 400 cc. of anhydrous ether and 15 cc. (0.2 mole) of dry pyridine was added. The mixture was cooled in a water bath and 14.0 Gm. (0.125 mole) of chloroacetyl chloride was added dropwise while the mixture was stirred. The resulting yellow-white precipitate was filtered and treated with 500 cc. of dilute acetic acid. The white residue obtained was recrystallized from ethanol and yielded 13.0 Gm. (55%) of white rod-shaped micro-crystals which melted at 274–275°, with sintering at 215–217° and resolidification at 225°.

Anal.—Calcd. for $C_{29}H_{20}Cl_2N_2O_3$: N, 5.61. Found: N, 5.58.

2,2′-Bis(diethylaminoacetamido)-9,9′-spirobifluorene.—Four and one-half grams (0.009 mole) of 2,2′-bis(chloroacetamido)-9,9′-spirobifluorene and 5.6 Gm. (0.077 mole) of diethylamine were refluxed in 1 L. of ethanol for twenty-four hours. The reac-

[1] Melting points are reported uncorrected.

tion mixture was cooled and poured into 3 L. of cold water. The resulting suspension of fine white precipitate was made alkaline with dilute sodium hydroxide solution, filtered, washed with water, and dried. The dry white powder obtained weighed 4.8 Gm. and melted at 235–241°. Recrystallization from ethanol yielded 4.5 Gm. (87%) of white micro-needles melting at 246–250°.

Anal.—Calcd. for $C_{27}H_{40}N_4O_2$: N, 9.78. Found: N, 9.77.

Table II contains two other analogous compounds which were similarly prepared by condensing piperidine and morpholine in place of diethylamine. The diisopropylamino analog could not be prepared by this method.

2,2′-Bis(*N,N*-dimethylcarbamido)-9,9′-spirobifluorene.—Ten grams (0.029 mole) of 2,2′-diamino-9,9′-spirobifluorene was dissolved in a mixture of 100 cc. of anhydrous benzene and 300 cc. of anhydrous ether. To this solution, there was added 8.0 cc. (0.1 mole) of anhydrous pyridine and 8.0 Gm. (0.075 mole) of N,N-dimethylcarbamyl chloride. The mixture was left standing for forty-eight hours with intermittent shaking. The red precipitate that formed was filtered, dried, and extracted with methanol. The methanol extract, upon treatment with water, yielded 2.0 Gm. (14%) of pale yellow micro-needles melting at 343°.

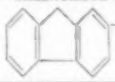
Anal.—Calcd. for $C_{31}H_{32}N_4O_2$: N, 11.49. Found: N, 11.25.

SUMMARY

1. Eight new aminoacyl derivatives of 2-aminofluorene and four new aminoacyl derivatives of 2,2′-diamino-9,9′-spirobifluorene are reported.

2. Hydrochlorides of a few representative samples of these new compounds were prepared and tested. They were found to exhibit some degree of antispasmodic activity on the isolated rat ileum. The tested materials also showed some local anesthetic action upon intradermal injection into the rat. They had little or no effect upon the rabbit cornea.

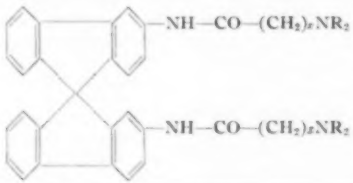
TABLE I.—AMINOACYL DERIVATIVES OF 2-AMINOFLUORENE



x	R₂	Formula	M. P., °C.[a]	Nitrogen, %[b] Calcd.	Found	Recryst. Solvents[c]	Yield, %
1	Diethyl	$C_{19}H_{22}N_2O$	125–126[d]	9.50	9.59	M + W	81
1	Diisopropyl	$C_{21}H_{26}N_2O$	124–125[e]	8.68	8.54	M—98%	35
1	Morpholino	$C_{19}H_{22}N_2O_2$	143–144[d]	9.07	9.09	M—50%	91
1	Piperidino	$C_{20}H_{22}N_2O$	134[f]	9.14	9.09	M—50%	78
2	Diethyl	$C_{20}H_{24}N_2O$	78–80[g]	9.08	8.96	M—50%	93
2	Diisopropyl	$C_{22}H_{28}N_2O$	181–182[h]	8.33	8.10	M—80%	40
2	Morpholino	$C_{20}H_{22}N_2O_2$	191–192[i]	8.69	8.81	M + W	93
2	Piperidino	$C_{21}H_{24}N_2O$	153–155[i]	8.73	8.56	M + W	93

[a] Uncorrected.
[b] Determined by a semi-micro Kjeldahl method.
[c] Recrystallizing solvents: M = methanol; W = water.
[d] Fine pale yellow needles.
[e] White micro-needles.
[f] White needles.
[g] Aggregates of twisted white plates.
[h] White microcrystalline powder.
[i] White rectangular plates.

TABLE II.—AMINOACYL DERIVATIVES OF 2,2′-DIAMINO-9,9′-SPIROBIFLUORENE



x	R₂	Formula	M. P., °C.[a]	Nitrogen, %[b] Calcd.	Found	Recryst. Solvents[c]	Yield, %
0	Dimethyl	$C_{31}H_{32}N_4O_2$	343(d.)[d]	11.49	11.25	M + W	14
1	Diethyl	$C_{27}H_{40}N_4O_2$	246–250[e]	9.78	9.77	E	87
1	Morpholino	$C_{27}H_{36}N_4O_4$	289[f]	9.33	9.24	E	84
1	Piperidino	$C_{29}H_{40}N_4O_2$	255[e]	9.39	9.30	E	75

[a] Uncorrected.
[b] Determined by a semi-micro Kjeldahl method.
[c] Recrystallizing solvents: E = ethanol; M = methanol; W = water.
[d] Pale yellow micro-needles.
[e] White micro-needles.
[f] White microgranular powder.

3. The hydrochlorides that were prepared for pharmacological testing were found to be only slightly soluble in water. This low solubility limits absorption and pharmacological action of these materials.

4. A summary is given of negative results in the attempted preparation of analogous aminoalkyl derivatives of 2-aminofluorene, 2,7-diaminofluorene, and 2,2′-diamino-9,9′-spirobifluorene.

REFERENCES

(1) Ray, F. E., and MacGregor, I. R., *J. Am. Chem. Soc.*, **69**, 587(1947).
(2) Cusic, J. W., U. S. pat. 2,527,798 October 31, 1950; through *Chem. Abstr.*, **45**, 2025(1951).
(3) Sargent, L. J., and Small, L., *J. Org. Chem.*, **13**, 608 (1948).
(4) Dahlbom, R., and Ekstrand, T., *Acta Chem. Scand.*, **4**, 578(1950).
(5) Weisburger, J. H., Weisburger, E. K., and Ray, F. E., *J. Am. Chem. Soc.*, **72**, 4253(1950).
(6) Kuhn, W. E., *Org. Syntheses*, **13**, 74(1933).
(7) Schulman, S., *J. Org. Chem.*, **14**, 382 (1949).
(8) Feldman, K., *J. Gen. Chem. (U. S. S. R.)*, **6**, 1234 (1936); through *Chem. Abstr.*, **31**, 1408(1937).

The Spectrophotometric Determination of Tetracaine and Phenylephrine Hydrochloride*

By ROBERT I. ELLIN† and ALBERT A. KONDRITZER‡

A method is presented for the quantitative determination of tetracaine and phenylephrine hydrochloride in therapeutic preparations based on ultraviolet spectrophotometry and colorimetry. Tetracaine absorbs light strongly in the spectral region of 310 mμ, while phenylephrine shows negligible absorption at this wave length. Phenylephrine is determined colorimetrically by a modification of a nitrosation method for phenols. Tetracaine is separated from its hydrolytic product, *p*-butylaminobenzoic acid, by extraction with water-saturated benzene below *p*H 3 and the concentration of tetracaine estimated in the aqueous solution, adjusted to a *p*H between 6 and 8, by measuring its optical density at 310 mμ. Phenylephrine solutions are treated with 15 per cent mercuric sulfate in 5 *N* sulfuric acid, heated on a water bath, cooled to room temperature, diazotized with 0.1 per cent sodium nitrite and diluted with distilled water. The red color developed is estimated in a colorimeter at 495 mμ and the concentration of phenylephrine determined by reference to a suitable standard.

THERAPEUTIC SOLUTIONS containing tetracaine and phenylephrine hydrochloride are known to deteriorate and lose their potency on standing. Tetracaine is subject to hydrolysis at the ester linkage while phenylephrine may undergo oxidation. For the development of stable preparations containing these medicinals, analytical methods were sought for their quantitative estimation which could be applied routinely with a minimum of manipulations to large numbers of experimental samples.

Relatively few methods for the quantitative determination of either tetracaine or phenylephrine have been described in the literature. Procaine, a compound structurally related to tetracaine, was determined photometrically by nitrosation and coupling with 2-naphthol (1). Bratton, *et al.* (2), coupled sulfanilamide with N-(1-naphthyl) ethylenediamine dihydrochloride and determined the resulting azo dye with a photoelectric colorimeter. This method has been adapted to the colorimetric estimation of procaine (3, 4). St. John (5) used ultraviolet absorption to determine procaine in the presence of penicillin G. Seydlitz (6) treated tetracaine with fuming nitric acid and determined quantitatively the nitro derivative by a colorimetric procedure.

Phenylephrine, being a phenol-type compound, would be expected to react analogously to phenols. Hanke and Koessler (7) devised a method for the quantitative colorimetric estimation of phenols by coupling with *p*-diazobenzenesulfonic acid in alkaline solution. Day and Taggart (8) brominated phenol and determined the quantity of aromatic compound from the amount of bromine consumed. Colorimetric methods for the determination of phenols and phenolic derivatives, based on the formation of a color when a phenol reacts with mercuric salts in the presence of nitrous acid, have been described by Folin, Lugg, and Arnow (9–11). Auerbach (12)

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† Professor of Pharmaceutical Chemistry, Rhode Island College of Pharmacy, Providence.
‡ With the technical assistance of John M. White and Irving Socholitzsky.

assayed phenylephrine by measuring the color intensity produced by the coupling of the drug with diazotized *p*-nitroaniline.

Since tetracaine and phenylephrine are aromatic compounds, they absorb ultraviolet light. As they differ markedly in their chemical structures, it was expected that they might exhibit ultraviolet absorption spectra which could be used as a basis for their estimation in the presence of each other. The application of absorption spectrophotometry showed that tetracaine absorbs ultraviolet light strongly in the region of wave length 310 mμ and much less at 228 mμ, while phenylephrine has maxima absorption bands at 215 and 273 mμ, but gives no measurable absorption at 310 mμ. It is thus possible to determine the concentration of tetracaine either alone or in the presence of phenylephrine by measuring its light absorption at 310 mμ, provided no interfering substances are present.

Tetracaine is the ester of dimethylaminoethanol and *p*-butylaminobenzoic acid (BABA). The property of absorbing ultraviolet light is due in most part to the presence in its molecule of BABA, which is liberated on hydrolysis. In order, therefore, to determine in aqueous solution the unhydrolyzed tetracaine in the presence of its hydrolytic products, it is more convenient to remove the BABA from solution. This can be done by extraction at an acid *p*H with an organic solvent immiscible with water. It was found that benzene quantitatively extracts BABA from aqueous solutions of *p*H below 3; with aqueous solutions of tetracaine below *p*H 3, none of the ester is extracted. Above *p*H 8 the results are reversed.

A colorimetric assay for phenylephrine was developed using a modification of Arnow's method for tyrosine. Although Auerbach's method for the determination of phenylephrine is more sensitive than the Arnow modification, the latter was used since it was more rapid and did not involve the preparation of a time-dependent diazo solution.

EXPERIMENTAL

Ultraviolet Absorption Studies.—Standard aqueous solutions containing tetracaine hydrochloride, 0.5 mg./cc., and phenylephrine hydrochloride, 0.5 mg./cc., were prepared. These solutions were diluted to 0.01 mg./cc. with distilled water and, using 1-cm. silica cells, spectral transmission curves between the wave lengths of 210 and 330 mμ were obtained with a Model DU Beckman Quartz Spectrophotometer (Fig. 1). Tetracaine has absorption maxima at 310 and 228 mμ, while phenylephrine has absorption maxima at 215 and 273 mμ, but gives negligible absorption at 310 mμ. Tetracaine can, therefore, be determined in the presence of phenylephrine by measuring absorption at 310 mμ.

Fig. 1.—Absorption spectra of tetracaine hydrochloride (curve *A*; 0.01 mg./cc.) and phenylephrine hydrochloride (curve *B*; 0.01 mg./cc.).

Optical density measurements at 310 mμ were determined on a series of solutions containing 0.25 to 2.0 mg. of tetracaine per 100 cc. The results, recorded in Table I, are in accord with Beer's law.

TABLE I.—RELATIONSHIP BETWEEN CONCENTRATION AND OPTICAL DENSITY OF TETRACAINE HYDROCHLORIDE

Concn. of Tetracaine Hydrochloride, Mg./100 Cc.	Optical Density	Concn., Mg./100 Cc.	Optical Density
0.25	0.170	1.47	
0.33	0.226	1.47	
0.50	0.340	1.47	
0.66	0.448	1.47	
0.75	0.510	1.47	
0.84	0.572	1.47	
1.00	0.681	1.47	
1.25	0.849	1.47	
1.35	0.916	1.47	
2.00	1.35	1.48	

Effect of *p*H on Ultraviolet Spectra.—In general, organic acids such as *p*-butylaminobenzoic acid can be separated from their ester derivatives by extraction from acid aqueous solutions. It was advisable, therefore, to determine the effect of hydrogen-ion concentration on the absorption spectra of tetracaine and of phenylephrine. Solutions of the drugs were prepared in 1 *N*, 0.1 *N*, 0.01 *N*, and 0.001 *N* HCl, acetic acid-acetate buffers of *p*H 4 and 5, dibasic sodium phosphate-dihydrogen potassium phosphate buffers of *p*H 6, 7, and 8, boric acid-potassium chloride-alkali buffers of *p*H 9 and 10 (13) and the absorption at 310 mμ measured. The results for tetracaine hydrochloride are given in Table II and show that the absorption is maximal and constant at *p*H's 4 to 9, but decreases sharply in more acidic and alkaline solutions. In the case of phenylephrine, absorption at 310 mμ remains negligible with change in hydrogen-ion concentration. Adjustment of the solutions to a neutral *p*H, e. g., approximately 6–8, for measurement of the optical density is indicated in order to eliminate possible errors due to lower or higher hydrogen-ion concentrations. There is no apparent shift in the spectral region of maximum absorption with change in *p*H.

TABLE II.—EFFECT OF pH ON ABSORPTION MAXIMUM OF 0.001 PER CENT TETRACAINE HYDROCHLORIDE

pH	Optical Density
~0	0.006
1.00	0.069
2.00	0.329
3.02	0.630
3.98	0.685
4.98	0.678
5.82	0.681
6.93	0.677
7.94	0.681
8.98	0.680
10.12	0.631

Separation of p-Butylaminobenzoic Acid from Tetracaine.—On standing, tetracaine hydrolyzes to BABA and dimethylaminoethanol. Absorption in the ultraviolet by the aminoalcohol is negligible. In order to determine the optimum pH for the separation of BABA from tetracaine, individual solutions of BABA, 0.0064 mg./cc., and tetracaine, 0.01 mg./cc., were prepared in the acid and buffer solutions described above. Aliquots of 20 cc. of solution at each pH were extracted with two 25-cc. portions of water-saturated benzene. The aqueous layer was filtered through dry filter paper, the first few cubic centimeters of the filtrate being discarded. A portion of the filtrate was adjusted to the pH range 6–8 and the optical density read at wave length 310 mμ. The results, plotted in Fig. 2, show that in solutions of pH below 3 all the BABA, but none of the tetracaine, was extracted, while above pH 8 all the tetracaine was extracted.

Determination of Phenylephrine Hydrochloride.—A modification of Arnow's method for tyrosine was used for the determination of phenylephrine hydrochloride. Aliquots of 1 cc. each, containing 0.3 to 1.0 mg. of phenylephrine hydrochloride solution were placed in test tubes graduated at 10 cc. To each tube was added 3 cc. of 15% mercuric sulfate in 5 N sulfuric acid. The contents were mixed, the tubes immersed in a boiling water bath for ten minutes, then cooled to room temperature. After addition of 3 cc. of 0.1% sodium nitrite, freshly prepared, the tubes were diluted to 10 cc. with distilled water. The solutions were then transferred to cuvettes and after fifteen minutes read in the Coleman Junior Spectrophotometer at 495 mμ. The red color developed was stable for at least fifty minutes. The calibration graph, Fig. 3, shows that the color intensity is in direct proportion to the concentration of phenylephrine within the range of concentration studied.

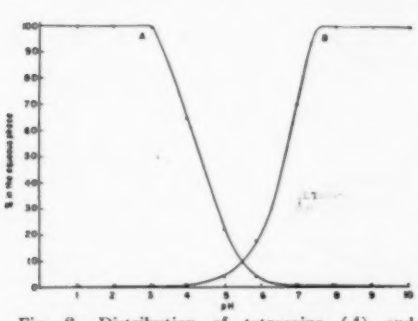

Fig. 2.—Distribution of tetracaine (A) and p-butylaminobenzoic acid (B) in the aqueous phase, when buffer solutions containing 0.01 mg./cc. of A or 0.0064 mg./cc. of B are shaken out with two 25-cc. portions of a water-saturated benzene solution.

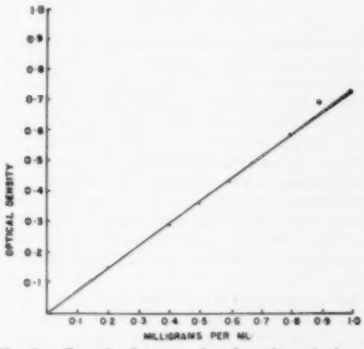

Fig. 3.—Standard curve showing the relationship of the color developed with phenylephrine hydrochloride to optical density at 495 mμ.

It should be noted that if glycols are present in tetracaine-phenylephrine solutions, heating in a boiling water bath after the addition of mercuric sulfate produces a precipitate. By allowing the sample to stand with both the mercuric sulfate and sodium nitrite, diluted to a volume of 10 cc. with distilled water at 30°, the precipitate may be avoided. After thirty minutes the color is maximal and may be determined in the spectrophotometer.

Procedure Recommended.—Prepare an aqueous solution from the experimental item to contain approximately 0.5 mg. of tetracaine hydrochloride and 0.5 mg. of phenylephrine hydrochloride per cc. If necessary, filter from any insoluble material and transfer, in duplicate, exactly 1 cc. to a test tube graduated at 10 cc. Add 3 cc. of 15% $HgSO_4$ in 5 N H_2SO_4 and proceed as described above for phenylephrine. After fifteen minutes the color is read in a colorimeter at 495 mμ and the concentration of phenylephrine determined by reference to a suitable standard.

For the estimation of tetracaine, dilute the aqueous solution fifty-fold with 0.01 N HCl. Extract a 20-cc. aliquot twice with 25 cc. of water-saturated benzene. Neutralize an aliquot of the aqueous filtered layer with an equal volume of 0.01 N NaOH and read the optical density with a spectrophotometer at 310 mμ. In the neutralized solution the concentration of tetracaine can be calculated from the following expression:

$$C_1 = \frac{C_2 D_1}{D_2} \qquad (1)$$

where

C_1 = concentration of unknown, mg./cc.

C_2 = concentration of tetracaine hydrochloride, 0.005 mg./cc.
D_1 = optical density of unknown.
D_2 = optical density of tetracaine hydrochloride, 0.005 mg./cc.

This method has been applied to the analysis of tetracaine-phenylephrine solutions, tablets, and concentrates. Deviations from the known composition were found to be less than 3%.

DISCUSSION

The spectrophotometric procedure described here for the estimation of tetracaine in the presence of phenylephrine includes an extraction step by means of which any liberated *p*-butylaminobenzoic acid is separated from unhydrolyzed ester, whose concentration is then estimated by measuring the optical density of the solution at 310 mμ. It is possible to carry out quantitative analyses by ultraviolet spectrophotometry on mixtures of the organic acid and ester without their separation since the absorption spectrum of BABA differs sufficiently from that of the tetracaine molecule. At 310 mμ, in the *pH* range of 6–8, 0.01 mg./cc. of tetracaine hydrochloride has an optical density of 0.681, while an equivalent concentration of BABA, 0.0064 mg./cc., has an optical density of 0.278. For a solution whose initial concentration of ester is known, the ratio of liberated acid to residual ester can be calculated by applying Beer's law to binary mixtures, as in the following equation:

$$\frac{R - K_{acid}}{K_{ester} - K_{acid}} \qquad (2)$$

where

R = determined optical density of the partly hydrolyzed sample diluted to x concentration with regard to the original tetracaine hydrochloride.
K_{acid} = a constant, the optical density of the *p*-butylaminobenzoic acid equivalent to x concentration of tetracaine hydrochloride.
K_{ester} = a constant, the optical density of tetracaine hydrochloride, x concentration, 310 mμ.

The presence of extraneous color caused by deterioration of the ingredients does not allow the use of this equation since the color interferes with the measurements of optical density. The extraction with benzene at acid *p*H removes the colored impurities as well as the BABA. In those solutions which show no discoloration, and especially in solutions containing only tetracaine as the therapeutic drug, the procedure on which equation 2 is based can be used.

SUMMARY

1. A spectrophotometric method for assaying tetracaine hydrochloride in the presence of phenylephrine hydrochloride is presented. At the absorption maximum of tetracaine, 310 mμ, phenylephrine showed no absorption.

2. An extraction method is described for separating tetracaine from its hydrolytic product, *p*-butylaminobenzoic acid.

3. The effect of hydrogen-ion concentration on the absorption spectrum of tetracaine is shown.

4. A colorimetric method for the analysis of phenylephrine has been established. The color developed is stable for fifty minutes. Recordings can be checked within that period of time without fear of color fading.

5. The procedures described are rapid and accurate. The potency and stability of tetracaine-phenylephrine preparations have been established spectrophotometrically.

REFERENCES

(1) Lapiere, C., *Anal. Chem. Acta*, **1**, 337(1947).
(2) Bratton, A. C., Marshall, E. K., Jr., Babbit, D., and Hendrickson, A. R., *J. Biol. Chem.*, **128**, 537(1939).
(3) Bendelin, F. J., and Kemp, C. R., *Ind. Eng. Chem.*, **18**, 470(1946).
(4) Graubard, D. J., Robertazzi, R. W., and Peterson, M. C., *Anesthesiology*, **8**, 236(1947).
(5) St. John, C. V., THIS JOURNAL, **37**, 343(1948).
(6) Seydlitz, H., *Svensk. Farm. Tid.*, **50**, 593(1946).
(7) Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, **50**, 235(1922).
(8) Day, A. R., and Taggart, W. T., *Ind. Eng. Chem.*, **20**, 545(1928).
(9) Folin, O., and Ciocalteau, V., *J. Biol. Chem.*, **73**, 627(1929).
(10) Lugg, J. W. H., *Biochem. J.*, **32**, 775(1938).
(11) Arnow, L. E., *J. Biol. Chem.*, **118**, 531(1937).
(12) Auerbach, M. E., THIS JOURNAL, **39**, 50(1950).
(13) Clark, M., "International Critical Tables," Vol. 1, McGraw-Hill Book Company, Inc., New York, 1926, pp. 81–84.

The Estimation of Some Analgetic Potencies*

By ARTHUR TYE† and B. V. CHRISTENSEN‡

A quantal method of analgesimetry was used to measure the analgetic activity of sodium acetylsalicylate, sodium pentobarbital, diethylaminoethanol, and the potentiating effect of sodium pentobarbital on codeine phosphate.

THE ANALGETIC POTENCY of a number of drugs was estimated by a quantal modification (1) of the Hardy-Wolff (2) method in which the Analgetic $Dose_{50}$ of the test drug is compared with the Analgetic $Dose_{50}$ of morphine in groups of rats subjected to a selected intensity of radiant heat stimulus during a fixed time interval. This method gave relative potencies for metopon (390), methadone (220), morphine (100), meperidine (24), and codeine (14) which correspond reasonably well with those reported clinically. Some experimental details of the method are here presented with the estimation of other analgetic potencies.

EXPERIMENTAL

A modified Hardy-Wolff-Goodell pain threshold apparatus was employed as the stimulator. This apparatus allows for the application of measured variable intensities of radiant heat for any desired interval of time. Two slight modifications of the apparatus were made. To prevent heat from passing by conduction through the metal tube between the source of heat and the exposure aperture, cold water was passed continuously through a coil of copper tubing which was wound around the full length of the tube. This eliminated any possibility of the test animal receiving stimulation from conducted heat. To prevent animals from being disturbed or conditioned by the click of the shutter that cut off the heat stimulus, a piece of asbestos tape was placed beneath the shutter to muffle the sound.

The animals used were a pure strain of Wistar white rat weighing between 100 and 200 Gm. They were housed and fed under uniform conditions and all work was done under uniform conditions of temperature and humidity. The tail of each rat was blackened with India ink and allowed to dry. The rat, lightly restrained by the operator's hand, was then placed on a platform so that the tip of its tail was across the aperture of the stimulator. Forcible restraint or confinement in a holder were found to be neither necessary nor desirable. (It was observed that the rats struggling against confinement often showed abnormally high pain thresholds.) Room temperature was kept at 20–24° C. and unusual stimuli such as loud sounds were avoided.

At the beginning of each experiment, the rats were subjected to a training period during which they were exposed for three seconds, at one-minute intervals, to stimuli of 450 millicalories, 320 millicalories, and 280 millicalories, in that order. If a rat failed to remove its tail at any one of these exposures, the stimulus was repeated at minute intervals until it responded. Only rarely did a rat require a prolonged training period.

For each drug three dose-groups were used; each consisting of twenty rats and with the logarithmically spaced doses arranged so that the expected percentage of analgesia in each group would be about 16, 50, and 84%, respectively. Each rat was tested for response forty minutes after subcutaneous injection of the drug. At that time the rat was exposed to a "reminder" stimulus of 450 millicalories for three seconds and thirty seconds later exposed to the test stimulus of 320 millicalories for three seconds. Its reaction as shown by movement of the tail, or failure to react, was noted. Failure to react was taken to indicate analgesia. This response was checked after another thirty-second interval. The data thus obtained were used to plot dose-per cent analgesia curves (Fig. 1). On the same day a curve for morphine was obtained in the same manner. Two days later the experiment was repeated as a crossover. The Wilcoxon and Litchfield (3) graphical procedure was used to test linearity and parallelism and for estimating the AD_{50}'s and the potency ratio with confidence limits.

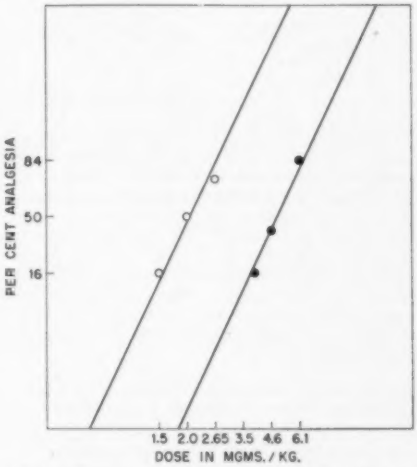

Fig. 1.—Dose per cent analgesia curves for morphine (●) and methadone (○) in white rats.

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† Assistant Professor, College of Pharmacy, Ohio State University.
‡ Dean of the College of Pharmacy, Ohio State University.

The AD_{50} of morphine sulfate was determined using female litter-mates in one series and males and females that were not litter-mates in another series. There was no significant statistical difference in the AD_{50}'s obtained (Table I). This agrees with Thorp's finding (4) that for analgesimetry, litter-mates are not superior to mixed rats from the same stock.

TABLE I.—MORPHINE AD_{50} IN FEMALE LITTER MATES AND MIXED RATS

Series	AD_{50} (19/20 Conf. Limits), Mg./Kg.	Potency Ratio (P. R.)
Mixed rats	4.7(4.3–5.2)	1.00
Litter-mates	4.4(3.9–4.9)	1.07[a]

[a] P. R. 1.07 is less than $f_{P.R.}$ 1.18; therefore the difference in potencies is not significant.

Morphine sulfate solutions of unknown strength were assayed by comparing their AD_{50} with that of a morphine sulfate solution of known strength. The estimated strengths were in good agreement with the actual strengths (Table II).

TABLE II.—ASSAY OF MORPHINE SOLUTIONS OF UNKNOWN STRENGTH

Solution	AD_{50} (19/20 Conf. Limits), Mg./Kg.	Potency Ratio (19/20 Conf. Limits)	Estimated % Strength (19/20 Conf. Limits)	Actual, %
Known	4.5(3.9–5.2)	1.0	...	0.1
Unknown No. 1	5.6(5.1–6.1)	0.8(0.7–0.9)	0.08(0.07–0.09)	0.07
Unknown No. 2	3.1(2.8–3.4)	1.5(1.3–1.7)	0.15(0.13–0.17)	0.16

Rovenstine (5) has pointed out that in man procaine is rapidly hydrolyzed to *p*-aminobenzoic acid and diethylaminoethanol (DEAE). Most of the acid is excreted unchanged, but the alcohol appears to be further metabolized. It has been suggested that DEAE is responsible for the analgetic effect of intravenous procaine. It was therefore thought worth while to test DEAE for analgetic activity. Freshly prepared aqueous solutions of DEAE were tested against morphine and methadone and found to have a very low analgetic potency as compared with them (Table III).

TABLE III.—ANALGETIC POTENCY OF DEAE AS COMPARED WITH MORPHINE AND METHADONE

Drug	AD_{50} (19/20 Conf. Limits)	Potency Ratio (19/20 Conf. Limits)
Morph. sulf.	4.7(4.2–5.2) mg./Kg.	1.0
Methadone HCl	1.9(1.6–2.2) mg./Kg.	2.5(2.2–2.8)
DEAE	1.1(1.0–1.2) cc./Kg.	0.0043 (0.0039–0.0047)

It was possible to produce an analgetic effect with DEAE, but only in toxic dosages. In the higher dose-groups, a number of animals died and all showed severe necrosis at the site of injection.

Sodium pentobarbital subcutaneously in doses up to 2 mg./Kg. was tested for analgetic effect. No analgesia was observed although the higher dosages caused pronounced sedation of the test animals. It was concluded that if sodium pentobarbital possesses any analgetic potency, it is not sufficient to raise the pain threshold in rats above 320 millicalories/sec./cm.[2]

The AD_{50} of codeine phosphate was compared with its AD_{50} when 1.5 mg./Kg. of sodium pentobarbital was administered simultaneously. No significant difference in the AD_{50}'s was observed, thus indicating that sodium pentobarbital has no potentiating effect on the analgetic activity of codeine phosphate (Table IV).

Freshly prepared aqueous solutions of sodium acetylsalicylate were tested for analgetic potency. No analgetic effect was observed with subcutaneous injections of doses up to 600 mg./Kg. It was concluded that sodium acetylsalicylate does not raise the pain threshold in rats above 320 millicalories/sec./cm^2.

TABLE IV.—COMPARISON OF AD_{50} OF CODEINE AND CODEINE PLUS PENTOBARBITAL

Drug	AD_{50} (19/20 Conf. Limits)	Potency Ratio (P. R.)
Cod. phos.	31.6(28.5–35.0)	1.00
Cod. phos. and pentobarbital	29.8(26.2–32.8)	1.06[a]

[a] P. R. 1.06 is less than $f_{P.R.}$ 1.16; therefore the difference in potencies is not significant.

DISCUSSION

A quantal method of analgesimetry yielded satisfactory estimates of the analgetic activity of potent analgetics and of the strength of morphine solutions, but it did not detect any analgetic effects with sodium pentobarbital and sodium acetylsalicylate. This does not necessarily mean that quantal threshold elevation methods are not sensitive enough to measure mild degrees of analgesia. The analgetic activity of barbiturates is open to considerable question and even if present does not appear to be susceptible to measurement by change in pain threshold (6, 7). The mild analgetic effect of acetylsalicylic acid is well recognized clinically, but pain threshold experiments have given conflicting results. Goetzl (8) concluded that the analgetic action of antipyretic drugs cannot be measured by threshold elevation, but other workers (2, 9, 10) claim positive results. Hardy, *et al.* (2), and Winder (9) obtained a maximal threshold rise of about 35% with acetylsalicylic acid. The method used in the present experiments was designed to measure the effects of potent analgetics which can cause a threshold rise of 50% or more. It is possible that by reducing the stimulus at which analgesia is measured to an appropriate level, for instance, 280 millicalories/sec./cm.^{2}, weaker analgetic effects such as those of acetylsalicylic acid could be measured by this method.

SUMMARY

1. Using a quantal modification of the Hardy-Wolff method of analgesimetry, satisfactory estimates of the strength of morphine solutions were obtained.

2. Neither pentobarbital nor acetylsalicylic acid were found to possess any analgetic activity.

3. Pentobarbital was not found to potentiate the analgesic effects of codeine.

4. Diethylaminoethanol was found to be analgetic only in toxic dosage.

5. Litter-mates were not found to be superior to mixed rats for analgesimetry.

REFERENCES

(1) Christensen, B. V., and Tye, A., THIS JOURNAL, **40**, 404(1951).

(2) Hardy, J. D., Wolff, W. G., and Goodell, H., *J. Clin. Invest.*, **19**, 649(1940).

(3) Litchfield, J. T., Jr., and Wilcoxon, F., *J. Pharmacol. Expll. Therap.*, **96**, 99(1949).

(4) Thorp, Roland H., *Brit. J. Pharmacol.*, **1**, 113 (1946).

(5) Rovenstine, E. A., *Ann. N. Y. Acad. Sci.*, **51**, 145 (1948).

(6) Andrews, H. L., and Workman, W., *J. Pharmacol. Expll. Therap.*, **73**, 99(1941).

(7) Winder, C. V., *Arch. intern. pharmacodynamie*, **74**, 176(1947).

(8) Goetzl, F. R., *Permanente Found. M. Bull.*, **4**, 49 (1946).

(9) Winder, C. V., *Arch. intern. pharmacodynamie*, **74**, 219 (1947).

(10) Bonnycastle, D. D., and Leonard, C. S., *J. Pharmacol. Expll. Therap.*, **100**, 141(1950).

Fungicidal and Fungistatic Evaluation of Certain Phenols and Surface-Active Agents*

By G. C. WALKER,† C. L. PORTER,‡ and H. G. DeKAY§

The antifungal activity of a series of phenolic chemicals and a number of surface-active agents is presented as determined on *Trichophyton mentagrophytes*, using 95 per cent ethanol as solvent. 2,4,5-Trichlorophenol and laurylpyridinium chloride appeared to be the most active of the compounds tested and are suggested as being worthy of clinical investigation in the therapy of tinea pedis.

THERE have been a number of publications concerning the fungicidal and fungistatic effectiveness of phenolic chemicals and the activity has been estimated primarily by methods involving conidial suspensions. During recent solvent investigations by the authors, a number of phenols were evaluated using the mycelial disk technique and employing as solvents 95 per cent ethanol and polyethylene glycol 400. The work reported here is an investigation of a more extensive series of phenolic chemicals and derivatives as well as a number of widely used surface-active agents for fungistatic and fungicidal activity using, respectively, the agar cup-plate test and the isolated disk technique. Certain commonly accepted antifungal compounds were included for our own purposes of comparison. It was desired to ascertain which compounds, if any, possessed marked antifungal properties with a view to using a phenol-surface-active combination with an antihistamine in the therapy of tinea pedis (1, 2), and as a screening prior to further investigations.

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† Associate Professor of Pharmacy, Ontario College of Pharmacy, Toronto, Ont., Canada.

‡ Professor of Botany, Department of Botany, Purdue University.

§ Professor of Pharmacy, School of Pharmacy, Purdue University.

EXPERIMENTAL

Fungistatic Evaluation.—The agar cup-plate method of Burlingame and Reddish (3) was adopted with modifications following those suggested by Taft (4). Serum agar was prepared by adding 10% sterile normal horse serum to Sabouraud's dextrose agar under aseptic conditions and pouring 20 cc. into 10-cm. Petri dishes. An additional 15 cc. of serum agar prepared as above was inoculated with 0.5 cc. of a conidial suspension of *Trichophyton mentagrophytes*[1] containing 5,000,000 conidia per cc. and the treated agar poured over the serum agar already in the plate. By means of a 2-cm. sterile cork borer a uniform hole was cut from the center of the agar plate and the cup sealed with a few drops of sterile agar. The chemical to be tested was dissolved in 95% ethanol and pipetted into the cup in quantities of 1 cc. The plates were incubated at 28 to 30° for five days and the zones of inhibition measured and recorded as the distance from the

[1] The authors wish to thank the Ontario Department of Public Health for supplying the organism used in this investigation.

nearest edge of the cup to the fungal growth. Control tests using 95% ethanol alone showed negligible zones of inhibition (5).

Larger zones of inhibition were produced using this method than were secured by simply streaking the plate with the test organism. At the same time, almost perfect circles of inhibition were achieved which rendered the measurement of zones more accurate. The results are shown in Table I.

RESULTS

Klarmann, *et al.* (6–9), have reported on the systematic investigation of the germicidal and fungicidal action of monohalogen-substituted phenol homologs in relation to the chemical constitution. The substituting halogen atom considerably intensified the microbicidal potency of phenol, substitution in the *para* position being more effective than *ortho* substitution. Introduction of alkyl groups into the nucleus of halophenols further increased their activity, the increase depending on the number of carbon atoms introduced.

Woodward, Kingery, and Williams (10, 11) investigated the fungicidal power of miscellaneous alkyl and halogenated phenols and found that halogen atoms increased the fungicidal power from four to ten times, the fungicidal strength increasing from chlorine through bromine to iodine. The introduction of nitro groups into phenol did not increase the fungicidal power. It was further pointed out that three times as much iodine and hexylresorcinol was required to kill the fungus in the presence of hide dust, and using blood serum, tenfold concentrations were required.

While the above results are principally fungicidal in nature, similar activity is manifested in the fungistatic tests outlined above.

From the limited number of tests performed by the agar cup-plate method, it appeared that halogenation of phenol and phenol derivatives provided compounds with enhanced fungistatic properties. Such increased activity was also manifested by the substitution of the benzyl, cyclohexyl, or phenyl group. Chlorine in the phenol ring was apparently more effective than bromine, and bromine more effective than iodine. The acyl phenols and esters showed little inhibitory tendency but an increase in activity was noted generally with an increase in length of the alkyl chain. 2,4-Dinitrocyclohexylphenol, *o*-aminophenol, and 2,4-dinitrophenol showed fungistasis only within the region stained.

TABLE I.—RESULTS OF FUNGISTATIC TESTS AT A CONCENTRATION OF 0.1% IN 95% ETHANOL USING *Trichophyton mentagrophytes*

Chemical	Clearance, Mm.	Chemical	Clearance, Mm.	Chemical	Clearance, Mm.
Control (95% ethanol)	0.5–1	2,4,6-Triiodophenol	6	2,4-Dimethyl-6-tert-butylphenol	6
o-Aminophenol	21	*o*-Methoxyphenol	[a]	2-Methoxy-4-propenylphenol	7
m-Aminophenol	1	*m*-Methoxyphenol	[a]	*p*-Ethylphenol	[a]
p-Aminophenol	[c]	*p*-Methoxyphenol	[a]	*o*-Propylphenol	13 [c]
o-Benzylphenol	19	2,3-Dimethoxyphenol	[a]	*o*-Butylphenol	19
p-Benzylphenol	20	*o*-Nitrophenol	1	*p*-Tert-butylphenol	18–5 [d]
2,4-Dibenzylphenol	5	*m*-Nitrophenol	20 [c]	2,6-Ditert-butyl-4-methylphenol	[a]
o-Bromophenol	1	2,4-Dinitrophenol	6	*p*-Tert-amylphenol	17
2,4-Dibromophenol	20	2,4,6-Trinitrophenol	1	Octylphenol-sec.	11
2,4,6-Tribromophenol	14	Phenol	[a]	Phenol Esters	
Pentabromophenol	5	*o*-Phenylphenol	19	Phenyl acetate	1
o-Chlorophenol	1	*p*-Phenylphenol	15	Phenyl butyrate	1
m-Chlorophenol	15 [c]	Acyl Phenols		Phenyl caproate	1
p-Chlorophenol	15 [c]	*p*-Hydroxyphenyl-methyl ketone	[a]	Phenyl caprylate	8 [c]
2,4-Dichlorophenol	[b]	*o*-Hydroxyphenyl-ethyl ketone	[a]	Phenyl caprate	9–3 [d]
2,4,5-Trichlorophenol	26	*p*-Hydroxyphenyl-ethyl ketone	[a]	Phenyl ester of phenylacetic acid	[a]
2,3,4,6-Tetrachlorophenol	15	*o*-Hydroxyphenyl-propyl ketone	[c]	Halogenated Substituted Phenols	
Pentachlorophenol	14	*p*-Hydroxyphenyl-propyl ketone	5 [c]	2-Bromo-4-tert-butylphenol	14–9 [d]
o-Cyclohexylphenol	16	*o*-Hydroxyphenyl-amyl ketone	3 [c]	2-Bromo-4-phenylphenol	12
p-Cyclohexylphenol	12	*p*-Hydroxyphenyl-amyl ketone	9	2-Chloro-*p*-hydroxytoluene	14 [c]
2,4-Dinitrocyclohexylphenol	7	Alkyl Phenols		6-Chloro-3-hydroxytoluene	14 [c]
o-Hydroxybenzaldehyde	[c]	*o*-Methylphenol	2	2-Chloro-*o*-phenylphenol	17
p-Hydroxybenzaldehyde	2	*m*-Methylphenol	[a]	4-Chloro-*o*-phenylphenol	18
o-Hydroxybenzoic acid	1	*p*-Methylphenol	1	3-Methyl-4-chloro-6-isopropylphenol	23–3 [d]
m-Hydroxybenzoic acid	[a]	3,5-Dimethylphenol	1		
p-Hydroxybenzoic acid	[a]	2,3,5-Trimethylphenol	11		
Salicylanilide	19				
o-Hydroxyphenol	[a]				
m-Hydroxyphenol	[a]				
p-Hydroxyphenol	2				
1,2,3-Trihydroxybenzene	5				
1,3,5-Trihydroxybenzene	[a]				
o-Iodophenol	[c]				

[a] No zone of inhibition.
[b] Clears the plate.
[c] Germination is evident and the plate gives indications of growth of the organism.
[d] Indicates a transition or intermediate zone of faint germination followed by clear agar to the cup.

TABLE II.—CHEMICALS SHOWING FUNGICIDAL ACTIVITY IN 95% ETHANOL AFTER AN EXPOSURE OF ONE MINUTE AT VARYING CONCENTRATIONS

Concentration		
1%	0.5%	0.3%
o-Aminophenol	2,4,6-Tribromophenol	2,4,5-Trichlorophenol
2,4,6-Tribromophenol	Pentabromophenol	2,3,4,6-Tetrachlorophenol
Pentabromophenol	2,4,5-Trichlorophenol	
2,4,5-Trichlorophenol	2,3,4,6-Tetrachlorophenol	
2,3,4,6-Tetrachlorophenol	Pentachlorophenol	
Pentachlorophenol	2,4,6-Triiodophenol	
2,4,6-Triiodophenol	2,4-Dinitrocyclohexylphenol	
Octylphenol-sec.	Octylphenol-sec.	
2-Bromo-4-phenylphenol		
2-Bromo-4-tert-butylphenol		
2,4-Dinitrocyclohexylphenol		
3-Methyl-4-chloro-6-isopropyl-phenol		

The introduction of the carboxyl, methoxyl, or hydroxyl group had apparently very little effect in producing fungistasis.

Surface-Active Agents.—Twenty commercial surface-active agents[2] were screened for fungistatic activity by the above method at a concentration of 0.1% in 95% ethanol. The most effective substance was laurylpyridinium chloride, which gave a zone of inhibition of 19 mm. The lauryl compound appeared superior to all those tested while bearing in mind the weaknesses postulated for this type of test procedure and the fact that the quaternaries generally evidence reduced activity (12).

Fungicidal Evaluation.—The chemicals previously screened for fungistatic activity were tested at a concentration of 1% in 95% ethanol for fungicidal activity using the test method of Golden and Oster (13) with a slight modification in the rinsing phase; following exposure, the disk was rinsed in 60% polyethylene glycol 400 for five minutes, then in 30% acetone for five minutes, and finally for three minutes in sterile broth and subcultured on sterile slants of Sabouraud's dextrose agar. Five of the surface-active agents were not completely soluble in 95% ethanol and the alcohol concentration was reduced to 50%. Observations for growth were final after twenty-one days.

The organism for all tests was *Trichophyton mentagrophytes*, the conidia of which withstood an exposure of ten minutes to a 1:70 aqueous phenol solution but not to a 1:50. For testing purposes the fungus was cultured for fifteen days on Sabouraud's dextrose agar and the agar cut into disks 1 cm. in diameter by means of a sterile cork borer. Control tests using 95% ethanol and immersion times up to ten minutes followed by thorough rinsing showed growth of the organism at all times of exposure. The results are shown in Table II.

None of the surface-active agents was effective at a concentration of 1% and an exposure of one minute, except laurylpyridinium chloride. This substance was also found fungicidal at 0.5% but not at 0.3%.

As pointed out by Golden and Oster (13), reduction in fungicidal activity is experienced with increasing dilution of the alcohol. Alcohol rapidly penetrates the fungous mat and agar disk, and for this reason is useful and at the same time a handicap, for it cannot be said that the above method of test is adequate in fungicidal screening since the alcohol and chemical will penetrate an agar disk and, depending on the chemical, exhibit fungistasis. However, if inhibition was the most important factor, then a substance such as 2,4-dichlorophenol which inhibits strongly should exhibit powerful fungicidal tendencies by this method. Our tests show that it possesses weak fungicidal activity.

SUMMARY

A study of the fungicidal and fungistatic data on a series of phenolic derivatives using the techniques outlined showed the halogenated compounds to be active and 2,4,5-trichlorophenol to be the most effective compound. Among the surface-active agents, laurylpyridinium chloride was particularly active and warrants further investigation.

REFERENCES

(1) Carson, L. E., and Campbell, C. C., *Science*, **111**, 689 (1950).
(2) Walker, G. C., DeKay, H. G., and Porter, C. L., THIS JOURNAL, **40**, 432(1951).
(3) Burlingame, E. M., and Reddish, G. F., *J. Lab. Clin. Med.*, **24**, 765(1939).
(4) Taft, E. F., *Drug Trade News*, **1939**, Dec. 19, p. 27.
(5) Oster, K. A., and Golden, M. J., THIS JOURNAL, **36**, 283(1947).
(6) Klarmann, E., Shternov, V. A., and Gates, L. W., *J. Am. Chem. Soc.*, **55**, 2576(1933).
(7) Klarmann, E., Gates, L. W., Shternov, V. A., and Cox, P. H., Jr., *ibid.*, **55**, 4657(1933).
(8) Klarmann, E., Shternov, V. A., and Gates, L. W., *J. Lab. Clin. Med.*, **19**, 835(1934).
(9) Klarmann, E., Shternov, V. A., and Gates, L. W., *ibid.*, **20**, 40(1934).
(10) Woodward, G. J., Kingery, L. B., and Williams, R. J., *ibid.*, **19**, 1216(1934).
(11) Kingery, L. B., Williams, R., and Woodward G., *Arch. Dermatol. and Syphilol.*, **31**, 452(1935).
(12) Quisno, R. A., Gibby, I. W., and Foter, M. J., THIS JOURNAL, **35**, 317(1946).
(13) Golden, M. J., and Oster, K. A., *ibid.*, **36**, 359(1947).

[2] The authors wish to thank the Rexall Drug Co. for supplying a number of the surface-active agents used in this investigation: Aerosol O. T., Areskap, Arlacel 83, Atlas G-2152, cetylpyridinium chloride, cetyltrimethylammonium bromide, diglycol laurate, Dispersol H. P., glycerylmonolaurate, Hyamine 1622, laurylpyridinium chloride, Miranol 45, polyethylene glycol dilaurate, Santomerse D, "Span 80," "Tween 80," triethanolamine, Triton X-100, Triton X-400, and Ultrapone S 100.

The Polarographic Characteristics of Phenylmercuric Nitrate*,†

By WALTER L. WUGGATZER‡ and JOHN M. CROSS§

The polarographic characteristics of phenylmercuric nitrate were determined in aqueous solutions over a *p*H range of 1.4 to 10.1 using various buffer systems with and without the addition of added electrolyte. Two waves were obtained in all cases. The first wave started from zero applied potential in solutions below *p*H 6.7 which did not contain KCl. The half-wave potential of the second wave was measurable in all solutions. In all instances, the higher the hydrogen-ion concentration, the more positive was the half-wave potential. The half-wave potential was also dependent upon the buffer used, the concentration of phenylmercuric nitrate, and the added electrolyte. Solutions buffered at *p*H 9.2 were measured quantitatively. The wave height varied proportionately with the concentration of phenylmercuric nitrate. The capillary characteristics are given.

THE POLAROGRAPHIC behavior of various inorganic compounds of mercury has been determined and reported in the literature. Among these compounds are mercuric cyanide (1–3), mercurous and mercuric nitrate (4), and complex iodides of mercury (5).

A search of the literature revealed only two publications regarding polarographic investigations of organic mercurials. The first of these was by Costa (6) who used various halides of alkyl mercury and one of aryl mercury in his work. The second was conducted on thiomersalate by Page and Waller (7). In the course of their work they also used phenylmercuric acetate and nitrate in acid solution.

In this investigation, the polarographic behavior of phenylmercuric nitrate is determined at various *p*H concentrations and a quantitative measurement of phenylmercuric nitrate is made polarographically.

After this work had been completed, Benesch and Benesch (8) published the results of their investigations of organic mercury compounds.

EXPERIMENTAL

The instrument used in this investigation was the Sargent Model XII photographic recording polarograph calibrated to read in microamps. All potentials were measured directly against the saturated calomel electrode. The dropping mercury capillary had the following characteristics: at a pressure of 57.3 cm. of mercury the droptime (t) on open circuit in 0.1 N potassium nitrate at 25° was 3.33 seconds, the weight of mercury dropping per second (m) was 2.33 mg., and $m^{2/3}t^{1/6}$ was 2.147.

The electrolysis cell of Lingane and Laitinen (9) was used. A small amount of gelatin was added to each solution to suppress any maxima that might occur in the wave. Dissolved oxygen was removed from all solutions by bubbling oxygen-free nitrogen through the sample for fifteen minutes. The entire assembly (electrolysis cell and dropping electrode) was kept in a constant temperature water bath maintained at 24° ± 1°. All samples were examined over a potential range of 0.0 v. to −1.53 v.

The buffer systems used for the determination of the polarographic characteristics of phenylmercuric nitrate were those of Britton and Robinson (10), Clark and Lubs (11), Kolthoff and Vleeschouwer (12), Kolthoff (13), and Michaelis (14). All buffers were made four times the concentration given in the references cited.

In this phase of the investigation, equal parts of a saturated solution of phenylmercuric nitrate (saturated at 25° ± 1°) and of the desired buffer were mixed. Thirty cubic centimeters of the resulting solution was placed in the electrolysis cell, nitrogen passed through the solution, and the polarogram recorded. The *p*H of the solutions was determined before and after electrolysis in the first few solutions. Since there was no change in *p*H after electrolysis, it was felt only one determination of *p*H need be made and this was done after electrolysis in the remaining solutions.

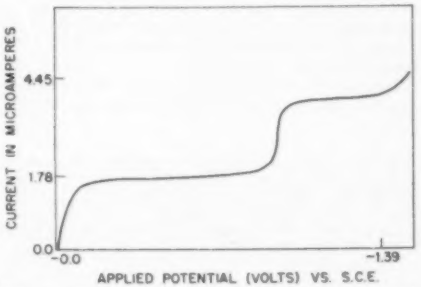

Fig. 1.—Typical polarogram of phenylmercuric nitrate in acid solution.

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† Abstract of thesis submitted to Rutgers University College of Pharmacy by Walter L. Wuggatzer as partial fulfillment of the requirements for the degree of Master of Science.

‡ Present address: Purdue University, College of Pharmacy, Lafayette, Ind.

§ Associate Professor of Chemistry, Rutgers University College of Pharmacy.

Figure 1 is a typical polarogram for phenylmercuric nitrate in acid solution; Fig. 2 is typical for alkaline solutions of phenylmercuric nitrate.

As part of this phase of the investigation, potassium chloride or potassium nitrate was added to a few of the solutions as a supporting electrolyte and solutions treated as before. The concentration of electrolyte in solution was 0.1 *N* in all cases.

All results are given in Table I.

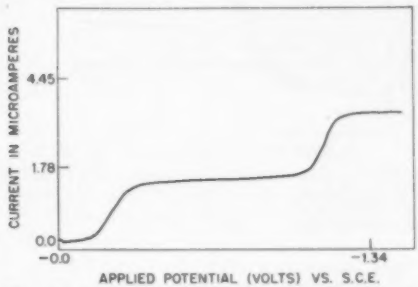

Fig. 2.—Typical polarogram of phenylmercuric nitrate in alkaline solution.

The half-wave potentials were found to vary considerably with the *p*H of the solution. Figure 3 illustrates this variance of $E_{1/2}$ with *p*H. This dependence of half-wave potential upon the *p*H of the solution may be attributed to the participation of hydrogen ions in the electrode reaction.

A plot of $E_{d.e.}$ *vs.* log $i/(i_d - i)$ does not give a straight line relationship for either the first or second wave which would indicate that the electrode reaction is irreversible.

TABLE I.—POLAROGRAPHIC CHARACTERISTICS OF PHENYLMERCURIC NITRATE

*p*H	Buffer System	$E_{1/2}$ *vs.* S. C. E. (Volts) 1st Wave	2nd Wave
1.5	B-R[a]	...	−0.780
2.8	B-R[a]	...	−0.861
3.7	B-R[a]	...	−0.896
4.6	B-R[a]	...	−0.981
5.5	B-R[a]	...	−0.984
6.7	B-R[a]	...	−1.063
7.3	B-R[a]	−0.132	−1.126
8.5	B-R[a]	−0.185	−1.144
9.4	B-R[a]	−0.225	−1.137
10.1	B-R[a]	−0.269	−1.159
2.8	B-R[a] KNO_3 added	...	−0.855
4.6	B-R[a] KNO_3 added	...	−0.930
6.7	B-R[a] KNO_3 added	...	−1.112
8.5	B-R[a] KNO_3 added	−0.181	−1.119
10.1	B-R[a] KNO_3 added	−0.254	−1.137
1.4	B-R[a] KCl added	−0.132	−0.473
3.6	B-R[a] KCl added	−0.139	−0.610
5.6	B-R[a] KCl added	−0.136	−0.769
7.5	B-R[a] KCl added	−0.136	−0.968
9.8	B-R[a] KCl added	−0.233	−1.223
1.4	C-L[b]	−0.149	−0.576
3.8	K-V[c]	...	−0.800
4.9	Kolthoff	...	−1.059
7.1	Michaelis	−0.247	−1.029
8.7	Michaelis	−0.368	−1.155

[a] Britton-Robinson buffer.
[b] Clark and Lubs buffer.
[c] Kolthoff and Vleeschouwer buffer.

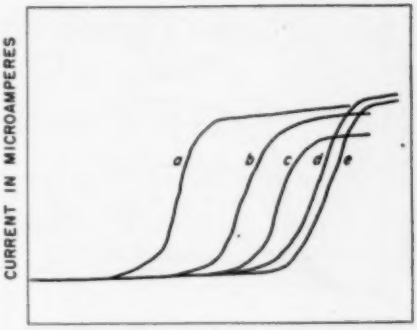

Fig. 3.—Effect of *p*H on half-wave potential of phenylmercuric nitrate in Britton-Robinson buffer.

(*a*) *p*H 2.8 $E_{1/2}$ −0.861 v.
(*b*) *p*H 4.6 $E_{1/2}$ −0.981 v.
(*c*) *p*H 6.7 $E_{1/2}$ −1.063 v.
(*d*) *p*H 9.4 $E_{1/2}$ −1.137 v.
(*e*) *p*H 10.1 $E_{1/2}$ −1.159 v.

For the quantitative determination of phenylmercuric nitrate, solutions of various concentrations of phenylmercuric nitrate were made up directly in twofold Britton-Robinson buffer *p*H 9.2. Thirty cubic centimeters of resulting solution was then treated as before and the polarogram recorded. Figure 4 illustrates the results obtained for the first wave; Fig. 5 the results for the second wave. Results are also given in Table II. The proportionality of the diffusion current to the concentration of phenylmercuric nitrate provides a method for its quantitative determination. The half-wave potentials of the second wave vary somewhat with concentration, i. e., the less concentrated the solution the more positive the half-wave potential.

SUMMARY AND CONCLUSIONS

Procedures are presented for the determination of the polarographic characteristics of phenylmercuric nitrate in various buffer systems with and without the presence of added electrolytes.

Phenylmercuric nitrate is reduced in two steps when studied over a potential range of 0.0 v. to −1.53 v. The half-wave potential of the first wave is not measurable in the more acidic solutions because it starts at zero applied potential but can be measured in solutions above *p*H 6.7.

TABLE II.—QUANTITATIVE DETERMINATION OF PHENYLMERCURIC NITRATE

Concn. of Phenylmercuric Nitrate, Gm./1000 cc.	First Wave $E_{1/2}$ Volts	First Wave Step Ht., mm.	Second Wave $E_{1/2}$ Volts	Second Wave Step Ht., mm.
0.6706	−0.215	65	−1.151	66
0.50295	−0.211	52	−1.148	52.5
0.3353	−0.211	34	−1.141	35
0.16765	−0.211	18	−1.123	18
0.06706	−0.196	7.5	−1.022	7

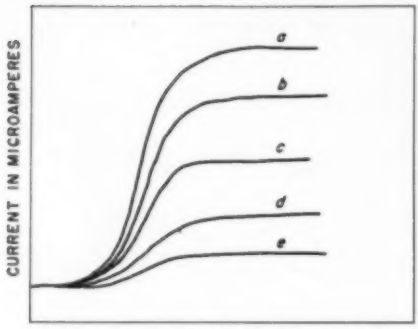

Fig. 4.—First waves—phenylmercuric nitrate in Britton-Robinson buffer *p*H 9.2. (*a*) 0.6706 Gm. (*b*) 0.50295 Gm. (*c*) 0.3353 Gm. (*d*) 0.16765 Gm. (*e*) 0.06706 Gm. in 1,000 cc. of buffer.

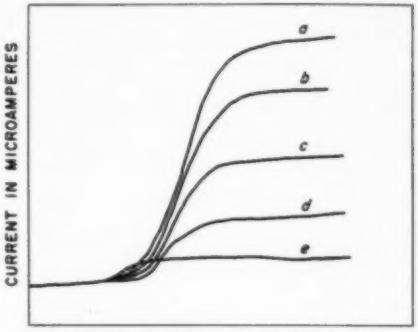

Fig. 5.—Second waves—phenylmercuric nitrate in Britton-Robinson buffer *p*H 9.2. (*a*) 0.6706 Gm. (*b*) 0.50295 Gm. (*c*) 0.3353 Gm. (*d*) 0.16765 Gm. (*e*) 0.06706 Gm. in 1,000 cc. of buffer.

The half-wave potential of the second wave is measurable in all solutions. The heights of both waves are equal. Increase in hydrogen-ion concentration causes the half-wave potential of both waves to become increasingly more positive.

Potassium nitrate as added electrolyte shifts the $E_{1/2}$ to a slightly more positive value.

Potassium chloride as added electrolyte precipitates the phenylmercuric as the chloride in solutions of low *p*H. The $E_{1/2}$ of both waves can be measured in the presence of potassium chloride. The $E_{1/2}$ of the first wave at the lower *p*H values is more negative as compared to the same solution without added potassium chloride. In a like manner, the $E_{1/2}$ of the second wave is more positive as compared with the same solution without added potassium chloride.

Quantitative determinations of phenylmercuric nitrate in aqueous solutions are feasible.

From all the evidence accumulated, it is felt that the mechanism of reactions involved in the electro-reduction of phenylmercuric nitrate is quite complex and no attempt is being made at this time to postulate a mechanism for this reduction.

The value of the half-wave potentials of phenylmercuric nitrate seems to be dependent mainly on the *p*H of the solution and to a lesser extent on the supporting electrolyte and the concentration of phenylmercuric nitrate in solution.

REFERENCES

(1) Dillinger, M., *Collection Czech. Chem. Communs.*, **1**, 638(1929).
(2) Kemula, W., *ibid.*, **2**, 347(1930).
(3) Tomes, J., *ibid.*, **9**, 81(1937).
(4) Kolthoff, I. M., and Miller, C. S., *J. Am. Chem. Soc.*, **63**, 2732(1941).
(5) Malyugina, N. I., Shchennikova, M. K., and Korshunov, I. A., *J. Gen. Chem. (U. S. S. R.)*, **16**, 1573(1946).
(6) Costa, G., *Ann. Chim. Applicata*, **38**, 655(1948).
(7) Page, J. E., and Waller, J. G., *Analyst*, **74**, 292 (1949).
(8) Benesch, R., and Benesch, R., *J. Am. Chem. Soc.*, **73**, 3391(1951).
(9) Lingane, J. J., and Laitinen, H. A., *Ind. Eng. Chem., Anal. Ed.*, **11**, 504(1939).
(10) Kolthoff, I. M., and Rosenblum, C., "Acid-Base Indicators," The Macmillan Co., New York, 1937, p. 262.
(11) Kolthoff, I. M., and Rosenblum, C., *ibid.*, p. 244.
(12) Kolthoff, I. M., and Rosenblum, C., *ibid.*, p. 254.
(13) Kolthoff, I. M., and Rosenblum, C., *ibid.*, p. 259.
(14) Kolthoff, I. M., and Rosenblum, C., *ibid.*, p. 258.

Preparation of Water-Soluble Metal Complexes of Rutin and Related Flavonols*

By CHARLES F. KREWSON and JAMES F. COUCH†, ‡

Because of the need for means of solubilizing rutin, a number of flavonoid metal complexes were prepared. Several of these, made from rutin in combination with iron compounds, appear to combine with rutin in a stoichiometric relationship. Particularly effective in solubilization of rutin is colloidal saccharated iron oxide. Solutions of rutin in saccharated iron oxide may be varied over an appreciable concentration range; they are stable, pleasant to taste, and should be tolerated by intravenous injection, since solutions of saccharated iron oxide alone are well tolerated.

THE EFFECTIVENESS of rutin in restoring increased capillary fragility and permeability in man to normal was established in 1943 by Griffith, Lindauer, and Couch and disclosed in a report to the Medical Society of the State of Pennsylvania (7), followed a year later by the publication of results (5).

Subsequently, rutin proved so effective that by 1946 over 1,000 case histories were available for evaluation. By using actuarial tables, it can be shown from these data (4) that when hypertension is complicated by capillary fault, patients not treated with rutin have a mortality rate 8.5 times normal, while patients treated with rutin have a mortality rate only 1.5 times normal. Treatment with rutin reduces the mortality rate due to apoplexy by about 90 per cent and that due to coronary occlusion by about 50 per cent.

The chemistry, pharmacology, and clinical applications of rutin have been summarized recently by Couch, *et al.* (3).

Usually rutin is administered to patients in the form of 20- or 50-mg. tablets, and the daily dose is 60 mg. (and upward). Because of its limited solubility (about 0.1 Gm. per liter in cold water) rutin has, of necessity, been given orally. Taken in this fashion it may require some time for the drug to be assimilated and become effective in restoration of capillaries to a state of reduced fragility. At any rate, it is not known how quickly orally administered rutin is assimilated, as an interval of three to four weeks must elapse before a repeat of the Gothlin test (for capillary fragility) is made (6). The need for rutin in soluble form is apparent from the number of requests received from industry and from physicians, especially physicians attending patients who have had gastrointestinal hemorrhage, for whom oral therapy may be considered inadvisable.

To date, the only published report of a water-soluble form of rutin found is that of Plungian (9) on the solubilization of rutin with methyl glucamine. In view of the need for water-soluble rutin for oral and injection purposes, further studies were initiated. The metal compounds given particular attention as possible solubilizing agents were those of iron, copper, manganese, and cobalt because of their added nutritional significance. *Nutrition Reviews* suggests situations where intravenous iron should be helpful (8).

For the most part, selection of various iron compounds for study was based upon therapeutic usage as indicated by inclusion in the United States Pharmacopeia or the National Formulary. Literature articles on the treatment of hypochromic anemia with ferrous gluconate administered either intramuscularly or orally (10) and with saccharated iron oxide intravenously administered (1, 2, 12) led to particular interest in these forms of iron as solubilizing agents for rutin.

EXPERIMENTAL

Solubilization of Rutin with Iron Compounds

A number of iron salts were tested with respect to their ability to solubilize or complex with rutin. The general procedure was as follows:

Purified rutin, $C_{27}H_{30}O_{16}\cdot 3H_2O$, N. F. IX (quercetin content, 0.8%), 0.70 Gm., was dissolved in approximately 150 ml. of boiling distilled water (almost a saturated solution). To boiling solutions of this strength were added varying amounts, from 0.25 to 4.00 moles, of iron compounds to be tested. After boiling about one minute, the solutions were filtered, cooled, each made to a volume of 150 ml., and the *p*H was determined. They were allowed to stand for twenty-four hours in stoppered bottles. At the end of this period, the solutions were filtered, and the residue was washed with portions of cold distilled water (total 5 ml.). The residue (unreacted rutin) was dried at 110° for several hours, then allowed to equilibrate with air to constant weight in

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‡ Deceased.

TABLE I.—EFFECT OF IRON SALT CONCENTRATION UPON RUTIN SOLUBILITY

Moles of Iron Compound per Mole of Rutin[a]	Iron Compound			
	$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$[b]	$FeCl_3 \cdot 6H_2O$	$Fe[CH_2OH(CHOH)_4COO]_2 6H_2O$	$Fe_4(P_2O_7)_3 \cdot 9H_2O$
	Weight of Unreacted Rutin, Gm.			
0.25	0.63	0.50	0.54	0.59
0.50	0.65	0.35	0.02	0.54
0.75	0.60	0.20	0.02	0.49
1.00	0.01	0.04	Trace	0.49
2.00	None	None	Trace	0.37
4.00	None	None	None	0.24
	pH of Filtrate			
0.25	3.2	2.7	3.7	5.6
0.50	3.2	2.4	3.7	5.2
0.75	3.2	2.3	3.9	5.2
1.00	3.3	2.1	3.9	5.2
2.00	3.3	1.9	4.2	5.2
4.00	3.4	1.6	4.2	4.9
	Weight of Iron Compound, Gm.			
0.25	0.10	0.07	0.13	0.24
0.50	0.20	0.14	0.25	0.48
0.75	0.31	0.21	0.38	0.72
1.00	0.41	0.28	0.51	0.96
2.00	0.82	0.56	1.02	1.92
4.00	1.64	1.12	2.04	3.84

[a] Rutin (0.700 Gm.) dissolved in 150 ml. of boiling water.
[b] Ferric ammonium sulfate [$Fe_2(SO_4)_3(NH_4)_2SO_4 \cdot 24H_2O$], 0.51 Gm. (0.50 mole), gave no unreacted rutin; pH of filtrate, 2.3.

order to compare its weight with that of original rutin used. Table I indicates that rutin was successfully solubilized by ferrous ammonium sulfate, ferric chloride, and ferrous gluconate, and only partially by ferric pyrophosphate. In addition to these, this same quantity of rutin was completely solubilized by 0.51 Gm. (0.50 mole) of ferric ammonium sulfate, $Fe_2(SO_4)_3(NH_4)_2SO_4 \cdot 24H_2O$, giving a solution of pH 2.3; and by 0.25 Gm. (1 mole) of ferrous lactate, $Fe(C_3H_5O_3)_2 \cdot 3H_2O$, N. F. V, giving a solution of pH 3.3.

It appears from these data that ferrous ammonium sulfate, ferric chloride and ferrous gluconate (and possibly ferric ammonium sulfate and ferrous lactate) bear a stoichiometric relationship to the rutin used. It would seem that rutin reacts in an equimolecular ratio with ferrous ammonium sulfate and ferric chloride, whereas 2 moles react with each mole of ferrous gluconate.

Several of these solutions (ones containing ferrous gluconate and ferric chloride) were examined spectrophotometrically. The maxima in the ultraviolet range were not markedly different from those of rutin or the iron compound involved, although slight spectral changes were observed in the visible range.

From the amounts of unreacted rutin obtained with ferric pyrophosphate (Table I), it appears that no complex is formed, although varying amounts of this compound do seem to have some solubilizing effect, influenced by the iron salt concentration.

Only when ferrous sulfate was combined as ferrous ammonium sulfate did it solubilize rutin. Ferrous sulfate alone, tried at different concentrations, failed to solubilize the flavonol. Data illustrating that combinations of ferrous sulfate and ammonium sulfate fail to solubilize rutin are presented in Table II. Other iron salts which failed to solubilize rutin when tried at different concentrations were: ferric sulfate, ferric ammonium citrate, ferric glycerophosphate, ferrous phosphate, and ferrous chloride. In the case of ferric ammonium citrate, different amounts of citric acid were added to determine whether change in pH might have any effect; there was none.

At about the time the work described above was completed, the previously mentioned article by Brown, *et al.* (2), on saccharated iron oxide came to our attention. Experiments have since been directed to the more practical aspects, such as methods of preparation of solutions containing possible therapeutic doses of rutin with ferrous gluconate and saccharated iron oxide.

Rutin-Ferrous Gluconate Preparations.—A stable solution of rutin and ferrous gluconate containing approximately 0.3 Gm. (N. F. IX usual dose) of

TABLE II.—EFFECT OF VARIOUS COMBINATIONS OF FERROUS SULFATE AND AMMONIUM SULFATE ON RUTIN SOLUBILITY 0.700 GM. OF RUTIN IN 150 ML. OF WATER

$FeSO_4 \cdot 7H_2O$		$(NH_4)_2SO_4$		pH of Filtrate	Weight of Unreacted Rutin, Gm.
Mole/Mole Rutin	Weight, Gm.	Mole/Mole Rutin	Weight, Gm.		
1	0.31	0.1	0.014	3.4	0.65
1	0.31	0.2	0.028	3.4	0.63
1	0.31	0.4	0.056	3.3	0.65
1	0.31	0.8	0.112	3.3	0.62
1	0.31	1.0	0.140	3.5	0.56
1	0.31	2.0	0.280	3.4	0.65
2	0.62	2.0	0.280	3.4	0.65

ferrous gluconate and 20 mg. (N. F. IX usual dose) of rutin per 5 ml. was prepared by dissolving 0.11 Gm. of rutin in 20 ml. of boiling water (saturated solution of rutin) and adding 1.50 Gm. of ferrous gluconate, N. F. IX (final concentration about 6% ferrous gluconate). The resulting dark-red solution was filtered while hot, cooled, and made to a volume of 25 ml. This solution had a *p*H of 4.1, was stable, and did not become moldy on storage in an unsterilized bottle for at least five weeks.

Similar solutions containing up to about 2.5% of dissolved rutin were prepared by this procedure; a 6% solution of ferrous gluconate as solvent was used. On standing for some time, a small amount of dark-green water-insoluble material appears, probably an oxidation product (11).

For convenience, rutin and ferrous gluconate may be mixed in powder form. For example, when the minimum amount of iron is desired, 0.55 Gm. of rutin can be mixed with 0.20 Gm. of ferrous gluconate. This mixture can then be dissolved in 100 ml. (measured cold) of boiling water. The dark-red boiling solution is filtered, cooled, and diluted to volume (100 ml.). The *p*H of the filtrate is 4.2; each 5 ml. of solution contains 28 mg. of rutin and 1.16 mg. of iron.

Rutin-Saccharated Iron Oxide Preparation.—A stable, aqueous colloidal rutin-saccharated ferric oxide preparation was made by dissolving 50.0 Gm. of saccharated ferric oxide, N. F. VII, (contained 3.1% iron) in 70 ml. of distilled water (*p*H of solution, 9.7). The volume became approximately 100 ml. when the solid was completely dissolved. When the solution was heated to boiling, different amounts of rutin, up to 6.0 Gm., could be dissolved. The solutions were filtered, cooled, and made to a volume of 100 ml., and their *p*H was measured. (Solutions so prepared may be diluted to any strength with water without precipitation of rutin.) Five milliliters (about a teaspoonful, a convenient amount for injection) of these solutions contained the average N. F. dose of 2 Gm. of iron compound and up to 550 mg. of rutin (N. F. average dose, 20 mg.).

Solutions prepared as described above are stable for at least six months when stored in stoppered glass bottles. Such solutions prepared with more than 1.5 Gm. of rutin occasionally set to a thixotropic gel.

The *p*H range of these solutions was from 6.8 (for 6.0 Gm. of rutin per 100 ml.) to 9.3 (for 0.40 Gm. of rutin per 100 ml.; this was a solution each 5 ml. of which contained 20 mg., the average N. F. dose). The *p*H of solutions containing 1.5, 2.0, and 3.0 Gm. of rutin was 8.0, 7.7, and 7.3, respectively. These preparations are sweet and pleasant to taste and do not have the usual astringent characteristic of iron compounds.

Solubilization of Rutin with Other Metal Salts

Copper.—A stable, aqueous, rutin cuprous chloride preparation was made by dissolving 0.70 Gm. of rutin in 150 ml. of boiling distilled water and adding 0.20 Gm. of the copper salt. The resulting deep orange-yellow solution containing the glycoside and metal salt in a mole ratio of about 1:2 had a *p*H of 4.0. (Unreacted rutin, 0.05 Gm. after twenty-four hours, was obtained from this solution, and the same amount of unreacted rutin was obtained from a 1:3 ratio.) One mole of cuprous chloride did not complex and 1 or 2 moles of cupric sulfate did not complex with 1 mole of rutin when treated under similar conditions.

Cobalt and Manganese.—Rutin in boiling solutions under conditions similar to those described above for copper, failed to complex with 1 and 2 mole ratios of cobaltous chloride, manganous chloride, or manganous sulfate.

Solubilization of Other Flavonols with Iron Compounds

Ferrous Gluconate Solutions as Solvents.—Quercetin ($C_{15}H_{10}O_7$) in any proportion failed to react with a 6% solution of ferrous gluconate.

Quercitrin ($C_{21}H_{22}O_{12}\cdot 2H_2O$), however, was solubilized by a boiling 6% solution of ferrous gluconate; one containing 1.50 Gm. of iron salt in 25 ml. of water dissolved up to 0.30 Gm. of quercitrin. The formation of considerable black solid (0.06 Gm.) on cooling made it appear impractical to proceed further with ferrous gluconate as a solubilizing agent for quercitrin.

TABLE III.—SOLUBILITY OF FLAVONOLS IN SOLUTIONS CONTAINING 50.0 GM. OF SACCHARATED IRON OXIDE PER 100 ML. OF WATER

Flavonol	Weight of Flavonol Soluble, Gm.	*p*H of Solution
Rutin	6.0	6.8
Quercetin	1.5	8.0
Quercitrin	3.0	7.5
Quercetagetin	2.0	7.7
Robinin	3.3	8.8

Saccharated Iron Oxide Solutions as Solvents.—Solutions of quercetin, quercitrin, quercetagetin ($C_{15}H_{10}O_7\cdot 2H_2O$) and robinin ($C_{33}H_{42}O_{20}\cdot 8H_2O$) were prepared in a manner similar to that described for rutin; 50.0 Gm. of saccharated iron oxide per 100 ml. of distilled water was used. Table III summarizes the information obtained. The quantity of flavonol indicated in each case is near the maximum solubility for that flavonol. These solutions were all stable for at least six months when stored in glass-stoppered bottles.

SUMMARY

Numerous iron salts were tested to determine their ability to increase the solubility of rutin in water. Ferrous ammonium sulfate, ferric chloride, ferrous gluconate, ferric ammonium sulfate, and ferrous lactate solubilized rutin by reacting in a manner which appears to be stoichiometric. Also, rutin was solubilized by colloidal saccharated iron oxide.

Ferric sulfate, ferric ammonium citrate, ferric glycerophosphate, ferrous phosphate, and ferrous chloride failed to solubilize rutin. Cuprous chloride, but not cupric sulfate, cobaltous chloride, manganous chloride, and manganous sulfate, solubilized rutin.

Ferrous gluconate failed to solubilize quercetin and quercitrin.

In addition to rutin, colloidal saccharated iron oxide solubilized quercetin, quercitrin, quercetagetin, and robinin.

Of the various metal combinations found effective in solubilizing rutin, colloidal iron oxide appears to offer the most promise.

REFERENCES

(1) Briscoe, C. C., *J. Am. Med. Assoc.*, **146**, 824(1951).
(2) Brown, E. B., Moore, C. V., Reynafarje, C., and Smith, D. E., *ibid.*, **144**, 1084(1950).
(3) Couch, J. F., Krewson, C. F., and Naghski, J., U. S. Dept. Agr., Bur. Agr. and Ind. Chem., AIC-291, February, 1951 (Processed).
(4) Griffith, J. Q., Jr., Philadelphia, Pa., personal communication. Data to appear in monograph on rutin now in preparation.
(5) Griffith, J. Q., Jr., Couch, J. F., and Lindauer, M. A., *Proc. Soc. Exptl. Biol. Med.*, **55**, 228(1944).
(6) Griffith, J. Q., Jr., and Lindauer, M. A., *Am. Heart J.*, **28**, 758(1944).
(7) Griffith, J. Q., Jr., Lindauer, M. A., and Couch, J. F., Medical Society State of Pennsylvania Program, 93rd Annual Session, October 5–7, 1943, p. 20.
(8) *Nutrition Revs.*, **9**, 92(1951).
(9) Plungian, M. B., U. S. pat. 2,451,772, October 19, 1948.
(10) Reznikoff, P., and Goebel, W. F., *J. Clin. Invest.*, **16**, 547(1937).
(11) Reznikoff, P., and Goebel, W. F., *J. Pharmacol.*, **59**, 182(1937).
(12) Scott, J. M., and Govan, A. D. T., *Lancet*, **1**, 367 (1951).

Studies on Species of *Asclepias*. IV. A Comparative Study of the Leaves*

By WILLIAM E. HASSAN, Jr.,† HEBER W. YOUNGKEN,‡ and MAYNARD W. QUIMBY§

This paper consists of a report on a study of the gross characteristics and histological features of leaves of *Asclepias tuberosa*, *A. syriaca*, *A. speciosa*, *A. incarnata* varieties, and *A. curassavica*.

THE PURPOSE of this paper is to report on a study of the comparative morphology of the foliage leaves of a number of species of *Asclepias*. Those considered are as follows: *Asclepias tuberosa* L., *A. syriaca* L., *A. speciosa* Torr., *A. incarnata* L. var. *pulchra* Hort., *A. incarnata* L. var. *alba* Hort., and *A. curassavica* L.

In the microscopical part of this investigation both temporary and permanent mounts were employed, these being prepared by the usual techniques.

The macroscopic and microscopic features of the leaves of *A. tuberosa* are described in considerable detail. However, the leaves of the other species are considered only in respect to their differences in comparison with the above-mentioned species (*A. tuberosa*) and with each other.

Internal phloem is present in the leaves of all species considered, at least in the larger portions of the midribs. A dorsiventral structure is exhibited by all of the leaves. Bundle sheaths are generally present and, in numerous instances, flanges of cells extend from the sheaths to the upper and lower epidermises.

All species examined, with the exception of *A. tuberosa*, contain latex.

For convenience and clarity the term "lamina" is employed in this paper to designate the flattened and expanded portions of a leaf located to either side of the midrib.

A report of a study involving the determination of vein islet number, palisade ratio, and stomatal index will be submitted later in another paper.

COMPARATIVE MORPHOLOGY

Asclepias tuberosa

The leaves of this species are alternately arranged on the stem, and are sessile or short-petioled. In general outline they vary from oblong to oblong-lanceolate. The apices are acute or obtuse, and the bases either rounded or somewhat cordate. These leaves vary in length from 5 to 15 cm. and in width from 0.6 to 2.5 cm.

Histology.—In transverse view the midrib appears as a broadly concavo-convex region elongated in the plane of the lamina and very narrow in its dorsiventral measurement. The upper epidermis of the midrib region consists of a single layer of small, ovoid to tangentially elongated cells possessing a thin cuticle and some epidermal hairs. The lower epidermis is somewhat wavy in this view and consists of cells which resemble those of the upper epidermis. Vascular tissue, including internal phloem, occurs as an elongated mass located approximately

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Abstracted from a portion of a thesis submitted to the Graduate Council of the Massachusetts College of Pharmacy by William E. Hassan, Jr., in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
† Instructor in Pharmacology and Biology, Massachusetts College of Pharmacy.
‡ Research Professor of Pharmacognosy and Botany, Massachusetts College of Pharmacy.
§ Professor of Botany and Pharmacognosy, Massachusetts College of Pharmacy.

in the center of the midrib. Among other histological features of the midrib region which should be mentioned is the presence of a single layer of collenchyma cells located adjacent to each of the two epidermises. Latex is not present in any part of this leaf.

The epidermises of the lamina portion each consist of a layer of tangentially elongated to somewhat ovoid cells interspersed by stomatal openings. A thin cuticle and some uniseriate, nonglandular hairs are also present on these layers.

In transverse sections the mesophyll is shown to consist of two zones, namely the palisade and spongy parenchyma. The palisade region is made up of either one or two layers of cells typical of such tissue. At intervals, the palisade zone is broken by the presence of flanges extending from the bundle sheaths. Spongy mesophyll of this leaf consists of large, thin-walled cells and conspicuous intercellular spaces.

In surface view the epidermal cells are large and have thin, wavy walls. Stomata present in the two epidermises possess from three to five neighboring cells. Numerous uniseriate, nonglandular hairs are present and may consist of from four to eight cells. These cells are rather thick-walled and are covered by "warty" protuberances.

Asclepias syriaca

These oppositely arranged leaves vary from oblong to oblong-ovate in outline, and are the largest of any of the leaves studied in this investigation. Their apices are acute to blunt, and their bases obscurely cordate. In length and width the leaves vary from 10 to 20 cm. and 5 to 10 cm., respectively. The petioles are averagely longer than those of *A. tuberosa*.

Histology.—The midrib of this leaf appears more or less semicircular in transverse section, the lower epidermis forming the convex margin. Vascular tissue appears in the upper third of the entire area. The cells of the epidermises of this region possess thicker walls than do corresponding layers in *A. tuberosa*. A thick cuticle occurs on the cells of the lower epidermis.

In the lamina portion the palisade region typically is made up of two layers of cells.

In surface view the cells of the upper epidermis are seen to be thin-walled and angular in outline. Most of the stomata of this layer possess four neighboring cells. Epidermal hairs are similar to those found on the leaves of *A. tuberosa* except that their cells are more nearly smooth. The lower epidermis of *A. syriaca* leaves, when examined in surface view, are shown to consist of small, wavy-walled cells. Most of the stomata possess four neighboring cells, but as many as seven such cells are present in some instances. Certain other stomata have three neighboring cells. Hairs are similar to those of the upper epidermis but are greater in number.

Asclepias speciosa

The leaves are distinctly petiolate and usually occur in pairs. However, the upper ones may sometimes appear in whorls of three. In length these leaves vary from 10 to 15 cm. and in width from 5 to 7.5 cm. They vary from broadly lanceolate to oblong-ovate in outline, and possess acute apices and tapering bases. Both surfaces may be gray-tomentose.

Histology.—In transverse section the midrib region is seen to have a rather distinctive outline. The margin formed by the upper epidermis is only slightly concave, while the lower margin is broadly convex, giving the section of the midrib a sub-ellipsoidal outline. An arc of vascular tissue appears near the center of the midrib. The lower epidermis consists of rather small squarish cells with moderately thickened walls.

Transverse sections of the lamina exhibit numerous nonglandular hairs on both epidermises. The palisade portion of the mesophyll consists of two layers of typical columnar cells. In the leaf material examined during this investigation no druses were observed.

An examination of surface sections of the upper and lower epidermises of the lamina shows them to possess very similar structure. The cells are angular (four- to six-sided) and appear to be covered by a striated cuticle. Nearly all of the stomata possess four neighboring cells. However, at intervals there occurs a stomatal apparatus with six such cells.

The epidermal hairs are nonglandular and uniseriate. Most of them are from two to five cells in length, but a few are more than five cells long.

Asclepias incarnata var. *pulchra* and *A. incarnata* var. *alba*

As might be expected, the leaves of these two plants have rather similar external characteristics. They are petiolate and their outlines vary from lanceolate to somewhat oblong-lanceolate. In the plants examined, the leaves of *pulchra* were shown to be averagely somewhat longer than those of the variety *alba*. Those of the first-named variety sometimes reach a length of 15.5 cm. Their epidermises are only slightly hairy.

Histology.—Examination of transverse sections shows that the midribs of these leaves are decidedly convex below and slightly concave above. The lower part projects well below the insertion of the lamina portions. The general outline in this view is broadly ovate. Vascular tissue is located in the upper half of the midrib.

The lamina portions of these leaves possess palisade zones of either one or two layers of cells typical of such a region. A few druses are to be observed in the lamina as well as in the midrib portion.

In surface view the cells of the lower epidermis of *pulchra* leaf are shown to possess wavy walls while the cells of the upper epidermis are angular in outline. Stomata show from three to six neighboring cells.

The epidermal cells of the variety *alba* are similar to those of the previously mentioned variety. Four to five neighboring cells are typical of these leaves.

Hairs are not abundant on any of these leaves. Most of those that do occur are found along the midrib and larger veins. These epidermal appendages are uniseriate and nonglandular, and some of them reach a length of nine cells. Hairs of the variety *alba* sometimes possess a greater over-all length than do those of *pulchra*, even when made up of the same number of cells. Hairs of these varieties are quite variable in general shape and wall thickness.

TABLE I.—A TABULATION OF SOME HISTOLOGICAL FEATURES OF THE LEAVES OF CERTAIN SPECIES OF ASCLEPIAS

	A. tuberosa	*A. syriaca*	*A. speciosa*	*A. incarnata* var. *pulchra*	*A. incarnata* var. *alba*	*A. curassavica*
Outline of midrib in T. S.	Broadly concavo-convex shallow dorsiventrally	Roughly semicircular; fairly deep	Sub-ellipsoidal	Broadly ovate	Broadly ovate	Roughly elliptical
Position of vascular tissue in midrib	Just above center	In upper third	Slightly above center	In upper half	In upper half	Just above center
No. of layers of pal.	1–2	Usually 2	2	1–2	1–2	2–4
Upper epidermis: cell outline	Wavy	Angular	Angular	Angular	Angular	Angular
Lower epidermis: cell outline	Wavy	Wavy	Angular	Wavy	Wavy	Angular
No. of neighboring cells	3–5	3–7; mostly 4	Usually 4; sometimes 6	3–6	4–5	4–6
Min. no. cells in hairs	4	4	2	4	2	2
Max. no. cells in hairs	8	9	Rarely more than 5	9	9	4

Asclepias curassavica

The leaves of this species are oppositely arranged on the stem. They are lanceolate to oblong-lanceolate, and possess acute apices and tapering bases. Hairs are present but are not numerous. Most of the pubescence occurs along the midrib and other veins.

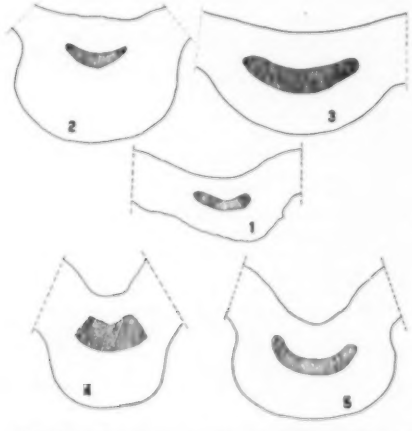

Fig. 1.—Transverse sectional diagrams of midribs of *Asclepias* leaves. Crosshatched portions represent vascular tissue. (*1*) *A. tuberosa;* (*2*) *A. syriaca;* (*3*) *A. speciosa;* (*4*) *A. incarnata* varieties; and (*5*) *A. curassavica.*

Histology.—In transverse section the outline of the midrib is quite characteristic. This region is broadly convex below and slight constrictions are evident below the points where the lamina arises laterally from the midrib. In this cross-sectional view the midrib is roughly elliptical in outline.

As many as four layers of cells sometimes occur in the palisade region of the lamina. However, two or three layers are more common. Only a moderate number of druses occur in the tissues of this leaf.

In surface view the cells of the upper and lower epidermises are angular in outline. The former cells are the larger. Stomata appear in both epidermises and possess from four to six neighboring cells.

The hairs are uniseriate and from two to four cells in length.

SUMMARY AND CONCLUSIONS

1. A report is given of the results of a morphological study of the mature foliage leaves of *Asclepias tuberosa, A. syriaca, A. speciosa, A. incarnata* var. *pulchra, A. incarnata* var. *alba,* and *A. curassavica.*

2. Druses were not observed in the *A. speciosa* material used in this study. Such crystals do occur in the other species considered.

3. Stomata occur in both epidermises of each of the plants examined.

4. *A. tuberosa* leaves are the only ones lacking in latex.

5. Transverse sections of the midribs are quite distinctive in outline, and this condition should be helpful in distinguishing these species from one another (Fig. 1).

6. Only the leaf of *A. curassavica* possesses a palisade zone of as many as three or four layers of cells.

7. In all species studied only uniseriate, non-glandular hairs were encountered.

8. A tabulation (Table I) of certain salient histological features is included as a part of this paper.

Numbers of Bacteria Removed from the Mouth as Measured by a Rinse Method*

By MORRIS OSTROLENK,† CHARLES E. MEYERS,‡ and MARIO MOLLARI§

Presented are the number of bacteria that can be removed from the oral cavity employing three rinse volumes, two media, and for two time periods. The results of successive washes and the examination of subjects for five weeks are correlated and discussed.

IT HAS BEEN stated by the Council on Dental Therapeutics of the American Dental Association (1) that "no method or combination of methods has been devised which will give a thoroughly satisfactory comparison of germicidal agents in a test tube and under the conditions in which they are actually used." Thus, there is need for *in vivo* methods for testing oral chemical germicides. Recent studies (2, 3), employing a wide variety of test organisms and chemical agents under several methods of test further emphasizes the inadequacy of *in vitro* tests as indicators of *in vivo* germicidal effectiveness. Goodrich (4), reporting on the apparent germicidal activity of three commercial mouthwashes drew conclusions from experiments in which glass cover slips and agar plates were used. Reddish (5) described refined *in vitro* antiseptic test methods which formed the basis of a report in *The Lancet* (6) on the activity of a commercial mouthwash, the investigator assuming that *in vitro* germicidal results could be correlated with *in vivo* activity.

With the development of standardized *in vitro* test methods (7), most of the subsequent investigators employed these techniques, or slight modifications. Thus, Bryan (8), Grubb (9), and Nolte (10), from *in vitro* tests, drew conclusions regarding the *in vivo* efficacy of the chemicals under test.

The studies reported here stemmed from a consideration of methods for *in vivo* evaluation of chemical mouthwashes. The evaluation of effectiveness of such washes must primarily rely upon securing representative samples of the oral microbial population before and after use of the washes. Therefore, the method of securing representative samples would appear to be important. Common to all such methods is the use of a substance or material for carrying the bacteria from the mouth. A variety of substances have been employed as vehicles for removing bacteria from the mouth. These include saliva (11, 12), paraffin (13), swabs (14), toothbrushes (15), and a variety of rinses (16–18). Objections can be raised to each of these: saliva depends upon the capacity of the individual to secrete per minute, and itself contains enzymes (lysozyme) and other antibacterial substances which *in vitro*, and undiluted, may produce an effect on organisms (19). Paraffin, according to Knighton (13), exerts a cleansing action upon the mouth and teeth; with swabs, the findings are apt to be dependent upon the area swabbed (20); and, with toothbrushes (used preliminary to rinsing), the effectiveness would be a function of individual skill which might increase during the test period.

For this work a rinse technique, utilizing physiological saline (0.85 per cent), seemed to be the simplest and easiest to control. The saline solution could be easily sterilized, the volume could be fixed with precision, its time of application could be governed; with "swishing" of the material, all portions of the oral cavity would be reached, most all individuals were equally skilled in its use, and even the most indolent subject could be persuaded to swish vigorously for thirty seconds.

The removal of microorganisms from the mouth was studied under several conditions. Investigated first was the effect of different volumes of the solution upon the number of organisms removed. Subsequent experiments will be described in the order they were performed. No more than an occasional comparison has been made between these findings and those of previous investigators such as Perjrone (17, 21), Turkheim (16), Appleton (18), and others. The dissimilarity in methods and techniques—Perjrone, for example, used three, pooled, 25-cc. washes for his calculations—makes such comparison useless, and at times misleading.

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Read at the 51st General Meeting of the Society of American Bacteriologists, May, 1951, Chicago, Ill.
† Bacteriologist, Food and Drug Administration, Federal Security Agency, Washington, D. C.
‡ Bacteriology Department, U. S. Naval Dental School, National Naval Medical Center, Bethesda, Md., and Georgetown University School of Dentistry. Commander (DC) USN.
The opinions or assertions contained herein are the private ones of Commander Meyers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.
§ Bacteriology Department, Georgetown University Medical and Dental School, Washington, D. C.
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MATERIALS AND METHODS

A "mouthwash" of sterile physiological (0.85%) saline was employed as the vehicle for removing microorganisms from the oral cavity. The volumes tested were 10–20 and 30 cc. To insure uniformity of procedure and to reduce potential experimental error, all dilution blanks were prepared from bulk sterile saline immediately prior to each day's test. Thirty-second oral rinse expectorates were collected in sterile Petri dishes, and without further mixing, were immediately diluted. The suspensions were shaken vigorously for ten seconds, as measured by an interval timer. Dilutions were planted in duplicate in agar containing 5.0% human blood, and in shake tubes of 0.4% thioglycollate semisolid agar.[1]

Approximately three and one-half to four minutes elapsed from the time of discharge of the expectorate until the final planting in the culture media. The inoculated blood agar plates and thioglycollate shake agar tubes were incubated for forty-eight hours at 34°. After incubation, counts were made and significant bacterial colonies selected for further study. Final numerical calculations were based on the total volume of the expectorates.

EXPERIMENTAL

Provision was made in the experiment design for the simultaneous investigation of several factors:

1. *Time of day:* Two periods: (1) morning, three to three and one-half hours after a meal; (2) afternoon, one to one and one-half hours after a meal.
2. *Culture media:* Two media: (1) 0.4% semisolid thioglycollate shake agar; (2) 5% blood agar (human blood).
3. *Volume of wash:* Three volumes: 10 cc., 20 cc., and 30 cc.
4. *Hemolytic streptococci:* The incidence of α- and β-streptococci.
5. *Subject variation:* Variations exhibited by the individual subjects.
6. *Interactions:* Any combination of the preceding factors.

Fifty-eight individuals participated in the experiment. Each washed his mouth with the three designated volumes of sterile physiological salt solution, a different volume being used on separate days. In addition, the subjects received the prescribed volume twice on a designated day—once in the morning and once in the afternoon. To avoid prejudice in recording, the first two of the three volumes were assigned according to a random sampling schedule, the third volume becoming fixed.

The experiment was conducted on three groups of 19, 18, and 21 individuals, respectively, designated as experiments 1, 2, and 3. The results of the experiments considered as a whole or by groups, and subjected to statistical analysis, confirmed several previously reported findings, differed from others, and disclosed a number of previously undemonstrated factors.

Time of Day.—With the exception of the first group, the time of day, with the accompanying difference in the length of time between eating and testing, had a substantial effect upon the numerical recovery of bacteria. The average for all three volumes combining both media, indicates that the morning counts were approximately 26% greater than the afternoon counts. A statistical analysis of variants disclosed that the difference in bacterial levels between morning and afternoon was significant. These findings are substantially in accord with those of other investigators.

Media.—The results revealed a not-altogether-unexpected, statistically significant difference in the productivity of the two media. Blood agar consistently gave higher bacterial counts than did semisolid thioglycollate shake agar. When compared with respect to the rinse-volume used and time of day, the relative productivity of the two media fluctuated but little; thioglycollate agar remained approximately 80% as productive as blood agar.

The procedure of culturing on two media and in duplicate made possible the determination of the respective standard deviations. The standard deviation between duplicate counts made on thioglycollate shake agar (13.1) is less than the standard deviation between duplicate counts made on blood agar (16.1).

The greater ease in planting of shake tubes of thioglycollate agar, together with the facility with which counts can be made are advantages supporting accuracy.

Volume of Wash.—Variation in the volume of the wash did not effect a significant change in the number of bacteria recovered. The results of the morning rinses for the three volume levels, cultured on thioglycollate agar, were 10 cc.—84 million; 20 cc.—89 million; 30 cc.—84 million. On blood the corresponding recovery values were 104–106 and 100 million bacteria. The bacteria counts obtained in the afternoon on thioglycollate agar were 10 cc.—59 million; 20 cc.—61 million; 30 cc.—61 million. On blood agar and in the afternoon, the corresponding numbers of bacteria recovered were 84–79 and 78 million of bacteria. Because of the shifting productivity between volumes on both media the differences between the mean of the bacterial counts are not significant.

Hemolytic Organisms.—Regardless of "time of day" (morning or afternoon), or "volumes" (10–20–30 cc.), the hemolytic colony estimates remained approximately 60% of the total bacterial counts. The ratio of hemolytic colony counts to the total bacterial counts for all subjects was 60.8% for the morning washes, and 60.7% for the afternoon washes. The close agreement in average percentage ratio of the hemolytic colony count to the total bacterial count for the different volumes were 10 cc.—63%; 20 cc.—58% and 30 cc.—59%, which also indicates the lack of effect of volume.

Subject Variation.—In an analysis of variance, a large variation was found in the number of bacteria recovered from the mouths of different subjects. The average bacterial counts of the subjects ranged from less than 10 million to more than 200 million. Although an analysis by subjects is not presented here, an inspection of the data revealed that some subjects washed out more microorganisms with one volume than with another. In addition, although the mean of the morning bacterial counts was significantly higher than the mean of the afternoon

[1] U. S. P. thioglycollate broth with 0.4% agar added. Medium tubed in 18 x 120 mm. tubes and sterilized in the conventional manner. The medium is liquefied in an Arnold steam bath and cooled to 45° before inoculation.

counts, some subjects shed more bacteria in the afternoon than in the morning. Similarly, although in almost every instance blood medium reproduced more microorganisms than thioglycollate medium, there were several exceptions due to subject interaction.

EFFECT OF REPEATED WASHING

Since analysis of the previous data did not indicate that a significant relationship existed between the number of bacteria removed and the rinse-volume used, and since it was the opinion of most subjects that a 20-cc. volume was both optimum and convenient for rinsing, this volume was employed in subsequent experiments.

Employing the previously described media and procedures, 20 additional subjects were examined twice daily (morning and afternoon) as follows:

(1) Each subject rinsed with 20 cc. of the test solution for thirty seconds, and emptied the solution into a sterile Petri dish;

(2) A second 20-cc. volume was immediately taken into the mouth for thirty seconds, and emptied;

(3) This procedure was continued for nine consecutive rinses with approximately a five-second interval elapsing between rinses;

(4) Rinses numbered 1, 2, 3, 5, 7, and 9 were cultured.

The average bacterial count for all subjects, and for all nine washes, showed a gradual diminution from the first through the seventh wash, i. e., 85, 74, 61, 49, 38, 40 million bacteria. However, the reduction was not the same for both media and was relatively greater for the blood agar count.

When the relative productivity of the two media was compared for all nine washes, it was seen that the thioglycollate agar count, expressed as a percentage of the blood media count, rose sharply in the morning, from 76 to 93%. In the afternoon, the percentage equalled or exceeded the blood agar count ranging from 100 to 117%.

The change in relative productivity of the two media strongly suggests the removal from the mouth of "transient" bacteria which are capable of growth on blood agar but not on the thioglycollate medium.

The incidence of hemolytic bacteria showed but little change from morning to afternoon. In the morning, the mean incidence of hemolytic organisms in relation to the total bacterial count was 49%; in the afternoon, it was 46%. The average for both morning and afternoon was 47.5%.

More detailed inspection of the results disclosed that according to the averages of the first wash bacterial counts, the subjects could be divided into three groups:

(*1*) *Low Group:* Subjects with first wash bacterial counts averaging less than 50 million.

(*2*) *Medium Group:* Subjects with first wash bacterial counts ranging from 50 million to 100 million.

(*3*) *High Group:* Subjects with first wash bacterial counts averaging more than 100 million.

The effects of the consecutive washes on the bacterial counts of each of the preceding groups was compared. It was seen that "Low Group" subjects showed a slight decrease during the first seven washes, and none thereafter. The counts of subjects falling in the "Medium Group" underwent a greater reduction through the first seven washes, with no significant change occurring thereafter. Subjects in the "High Group" showed a rapid reduction through the seventh wash and no further reduction.

STABILITY OF ORAL BACTERIAL COUNTS

Since the data in the previously described experiments indicated that there was a wide variation in numbers of bacteria that could be washed from the mouth of different subjects, an inquiry in regard to the stability of the bacterial count of individuals seemed indicated. Sixty-three subjects were each examined once according to the previously described technique. Based on these results, 30 subjects meeting the previously defined criteria of "High," "Medium," and "Low," were selected for further study. This placed 10 subjects in each one of the three groups. The subjects were examined once a week for five weeks.

An analysis of the data revealed that there were no distinct time trends over the five-week test period. The average thioglycollate bacterial counts from subjects in the "Low," "Medium," and "High" groups were 28.8 million, 66.6 million, and 152 million, respectively. On blood agar the corresponding counts were 39.2 million, 82.7 million, and 198 million. In both media the smallest amount of subject variation in bacterial counts occurred in the "Low" group, the standard deviation between subjects ranging from 21 to 85 for thioglycollate agar, from 24.2 to 109 for blood agar for the "Low" and "High" groups, respectively.

The standard deviations between duplicate tubes or plates of the two media employed revealed that the difference in count between duplicate cultures was largely dependent upon the magnitude of the count. With thioglycollate agar, a fivefold increase in the average bacterial count (from 28.8 million, "Low" group, to 152 million, "High" group) brought an almost fourfold increase in the standard deviation. Comparable results were obtained with blood agar.

The average hemolytic colony count for each of the three groups expressed as percentages of the total count on blood agar, was approximately 60, 66, and 64% for the "Low," "Medium," and "High" groups, respectively. This indicates that there was no appreciable difference in the interrelationship between the hemolytic colony count and total blood agar count for the three groups.

DISCUSSION

In order to correlate the findings of these apparently diverse experiments, it becomes necessary that they be viewed from the perspective of a common reference point. It has been stated previously that the experiments primarily stemmed from a consideration of establishing methods for evaluating oral chemical mouthwashes. However, before these agents can be rationally appraised and subjected to test, a more precise knowledge must be had of some aspects of the microbial flora which the agents aim to affect.

All experiments showed the subject to be one of the factors responsible for the greatest amount of variation.

In particular, the volume studies demonstrated that if the results were to be considered on the basis of individuals, or anything less than a series of tests, they would appear discordant. Statistically, variations of the magnitudes encountered in these tests are to be overcome only by repeated series of tests, or large numbers of subjects, or both. However, despite the wide variation in results between subjects, definite patterns evolved in which reliability can be placed.

Statistical evaluation of the data of the volume studies for all 58 subjects, comprising approximately 1,400 tests, makes it evident that any effect exerted by the volume of a rinse upon the number of bacteria delivered by the rinse could just as likely have occurred due to chance. In these studies, the results obtained in regard to the relative productivity of the two media, the effect of time (of day) of test, and the hemolytic-total-count ratio were statistically significant. Although each of the two media was expected to support the growth of different types and numbers of bacteria, with but one exception which occurred in the consecutive rinse studies, a reasonably consistent numerical relationship was seen to prevail. Thioglycollate agar remained approximately 80% as productive as blood agar. The comparatively minor departures from this consistent percentage that were observed to occur from morning to afternoon should be considered in regard to the consecutive wash study departures from this percentage. Parenthetically, it should be stated that the consecutive wash studies are not yet complete; the sample size is as yet too small to permit positive conclusions to be drawn. However, it is felt that the trends so far discerned possibly make its incorporation in this report of some value to other interested investigators.

It has been shown previously that the time of day (22–24), or the abrasive action of food (13), or both, markedly affect the number of bacteria recoverable in mouth rinses. These experiments permit it to be more precisely stated that in a sample composed principally of young, adult males, approximately 26% more microorganisms are recoverable in the morning than in the afternoon. In addition to this, it may be stated that whatever changes occur in the total count due to "time," or "food," or both, do so without in any way altering the hemolytic to total-count ratio. Sixty per cent of all the bacteria that were removed from the mouth both morning and afternoon, and by all three volumes, were α- or β-hemolytic types.

In the consecutive wash studies, upon which work is continuing, the rate of reduction in bacterial counts seemed to reflect the category of the patient; i. e., "high," "medium," or "low." However, all subjects, regardless of category, reached a plateau with the seventh wash, after which no further reduction in count occurred. It was observed that on blood agar and in the morning, the plateau coincided with a reduction in count of 63%; in the afternoon the comparable figure was 44%. In the modified thioglycollate shake agar, the plateaus were reached when the precentages were 55% in the morning, and 38% in the afternoon.

Although in the volume studies the thioglycollate agar was approximately 80% as productive as blood agar, in the consecutive wash studies this relationship was observed to change with rinsing. On the first morning wash, thioglycollate agar was about 80% as productive as blood agar. With succeeding washes, however, the relative productivity of thioglycollate agar increased and by the seventh wash was 93% as productive as blood agar.

For the series of afternoon washes, the relative productivity of the two media is about equal. This contrasts with the volume studies where the superiority of the blood agar was slightly more pronounced in the morning than in the afternoon. The change in relative productivity is evident in the afternoon series, however, with thioglycollate agar equal to blood agar on the initial wash and reaching 117% of the blood agar count on the ninth wash. An explanation for the changes in the relative productivity of the two media occurring in the consecutive wash studies obviously must lie in the early removal by the initial washes of specific types of microorganisms particularly disposed to grow on blood agar. This, in turn, suggests more fundamental concepts (25), such as "transient," and "resident" oral microorganisms, and the manner in which this flora may be "layered."

Although the reverse ratio of productivity observed to occur between the two media during the course of the consecutive rinses demonstrated that an unknown flora was being removed, the proportion of hemolytic organisms did not follow this pattern. The evidence appears to indicate that the hemolytic organisms did not comprise the major part of the unknown flora capable of growth on blood agar that the consecutive washes removed. The hemolytic organisms must constitute a rather consistent proportion of the total oral flora both "transient" and "resident."

During the course of the consecutive washes, it became evident that the subjects could be roughly grouped on the basis of the number of bacteria removed by rinses. The classification of 30 subjects into three groups—in "low," "medium," and "high" count categories on the basis of a single rinse test—and the prolonged examination of these subjects for five weeks revealed there to be no distinct time-change trend. The "low" group showed the least amount of variability which progressively increased through the "medium" and "high" groups. Such factors may be of particular significance when viewed in connection with the development of methods for *in vivo* evaluation of oral chemical agents.

SUMMARY

The primary purpose of this study was to investigate some fundamental aspects of oral bacteriology of ultimate value in testing the effectiveness of oral chemical "mouthwashes."

A rinse technique with physiological salt solution was employed for the removal of microorganisms from the mouth.

Variation in the volume of rinse solution from 10 to 20 and 30 cc. did not influence the number of bacteria removed from the mouths of the test subjects. Approximately 26 per cent more microorganisms were removed by morning rinses than by afternoon rinses from the same test sub-

jects. Rinse counts on thioglycollate shake agar consistently represented about 80 per cent of the number of bacteria growing on blood agar. About 60 per cent of the total number of bacteria growing on blood agar were α- or β-hemolytic types without regard to time of day, volume of wash, or subject variation. Subjects varied considerably in the number of bacteria recoverable from the mouth, with counts ranging from less than 10 million to more than 200 million.

In these experiments, seven consecutive washes reduced the number of bacteria recoverable from the mouth to what appeared to be an irreducible minimum. The relative reduction in bacterial numbers produced by consecutive rinses was a function of the initial bacterial counts of the subjects.

The number of bacteria that can be removed from the oral cavity of a subject by means of a rinse did not reveal any distinct time trends.

REFERENCES

(1) "Accepted Dental Remedies," 16th ed., American Dental Association, Chicago, Ill., 1951, p. 138.
(2) Ostrolenk, M., and Brewer, C. M., THIS JOURNAL, **38**, 95(1949).
(3) Ostrolenk, M., *ibid.*, **39**, 71(1950).
(4) Goodrich, H. P., *Brit. Med. J.*, **2037**, 473(1917).
(5) Reddish, G. F., *J. Lab. Clin. Med.*, **14**, 649(1929).
(6) "Reports and Analytical Records," *Lancet*, **217**, 616 (1929).
(7) Ruehle, G. L. A., and Brewer, C. M., "U. S. Food and Drug Administration Method of Testing Antiseptics and Disinfectants," U. S. D. A. Circ. No. 198, 1931.
(8) Bryan, A. H., THIS JOURNAL, **27**, 654(1938).
(9) Grubb, T. C., *ibid.*, **36**, 385(1947).
(10) Nolte, W. A., Edwards, M. A., and Grubb, T. C., *ibid.*, **38**, 258(1949).
(11) Hadley, F. P., *J. Dental Research*, **13**, 415(1933).
(12) Slanetz, L. W., and Brown, E. A., *ibid.*, **28**, 313(1949).
(13) Knighton, H. T., *J. Am. Dental Assoc.*, **29**, 2017 (1942).
(14) Bloomfield, A. L., *Bull. Johns Hopkins Hosp.*, **33**, 145(1922).
(15) Snyder, M. L., *J. Dental Research*, **18**, 497(1939).
(16) Turkheim, H. J., *Zahnärztl. Rundschau.*, **31**, 1205 (1930).
(17) Perjrone, G. M., *Dental Cosmos*, **77**, 800(1935).
(18) Appleton, J. L. T., *ibid.*, **69**, 1026(1927).
(19) Appleton, J. L. T., "Bacterial Infection in Dental Practice," 4th ed., Lea and Febiger, Philadelphia, Pa., 1950, p. 420.
(20) Arnold, L., and Stuart, C., *Am. J. Digestive Diseases*, **2**, 275(1935).
(21) Perjrone, G. M., *Stomatolgiya*, **11**, 1014(1934).
(22) Appleton, J. L. T., "Bacterial Infection," 3rd ed., Lea and Febiger, Philadelphia, Pa., 1944, p. 316.
(23) Feirer, W. A., and Leonard, V., *Dental Cosmos*, **73**, 338(1931).
(24) Appleton, J. L. T., "Bacterial Infection," 3rd ed., Lea and Febiger, Philadelphia, Pa., 1944, p. 306.
(25) Price, P. B., *J. Infectious Diseases*, **63**, 301(1938).

The Physics of Tablet Compression. I. A Preliminary Report*,†

By T. HIGUCHI, R. D. ARNOLD,‡ S. J. TUCKER,§ and L. W. BUSSE

A preliminary report surveying a series of basic studies on compressed tablets is presented. For procurement of data toward elucidation of tablet structure and behavior, tentative methods for determining (*a*) tablet density, (*b*) bulk density of the mixed ingredients, (*c*) porosity and pore size of tablets, and (*d*) the compressional force and compressional work necessary for formation of tablets have been developed. Other methods for obtaining additional dynamic and static information related to formation of tablets are also discussed. No attempt is made at this time, however, to correlate the data obtained with tablet performance.

THIS PAPER constitutes a preliminary report of basic studies on compressed tablets which have been in progress in these laboratories. The manufacture of compressed tablets has been the object of intensive investigation since early in the 19th century. Most of the studies in this field, however, have centered around the solution of the practical problems encountered in the compression of a wide variety of drugs into tablets. Much empirical information is available on the relative efficacies of different lubricants, binders, fillers, and disintegrating agents in various combinations, but no serious attempt has been made to correlate such physical factors as porosity, density, hardness, crystalline structure, compressibility, etc., of both the tablets and tablet ingredients. An introduction to an attempt in this direction is presented at this time.

Considerations of tablets formed by compression can advantageously be divided into two

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‡ Present address: Fort Sam Houston, Tex.
§ Present address: Merck and Co., Rahway, N. J.

types; namely (*a*) static ones related to the pore size, the porosity, the tensile strength, the cohesive forces involved, the modulus, etc., of the tablets, and (*b*) the dynamic, energetic considerations of pressures of formation, rates of compression, work of compression, frictional energies involved, etc. These factors, in turn, must be correlated, in the final analysis, with the disintegrating characteristics of the formed tablets.

Tentative experimental methods of evaluation of each of these factors are outlined in the following sections. No attempt is made, however, to present a full report of the experimental data obtained or their analysis. A more detailed account of each topic will be published later.

STRUCTURE OF TABLETS: STATIC CONSIDERATION

Compressed tablets generally consist of a variety of substances having different physical properties which contribute to the general structure of the tablet. The active medicament, e. g., may be organic or inorganic, a salt or an acid, an ester or an alcohol, all of which may have different crystal structure and properties which will influence the compression characteristics of a formula. Binding agents are usually carbohydrate or proteinaceous in character. All the many agents employed such as acacia, tragacanth, sugar, dextrin or starch paste, gelatin and the percentage in which they are used influences the compression characteristics of a formula. The physical properties of the fillers employed such as dextrose, calcium sulfate, sodium chloride, dextrin, kaolin, magnesium oxide, and aluminum hydroxide and their ratio to the total formula exert a great influence on the compression characteristics of any formula. Likewise the disintegrating agent and its physical properties and structure in addition to the amount present in the formula will have a great influence. The lubricants used such as talc, soaps, mineral oil, boric acid, etc., influence the binding characteristics of these substances when they are subjected to compression. Combinations of these many substances in their proper proportion when compressed will yield a tablet possessing a certain structure which may be varied according to the physical properties and particle sizes of the ingredients. As the structure of the tablet is changed, so will the disintegration rate be affected.

The elucidation of the structure of tablets will necessarily be based in part on the following factors: (*a*) bulk density of the granulation and the density of the tablet, (*b*) pore size, and (*c*) the cohesive strength and hardness.

Determination of the Porosity of Tablets.—Although no substantiating experimental data are available, it seems reasonable to assume that the volume of void space in a given tablet would greatly influence its disintegrating property. For this reason an attempt was made to develop a suitable method for determining this quantity for tablets in general.

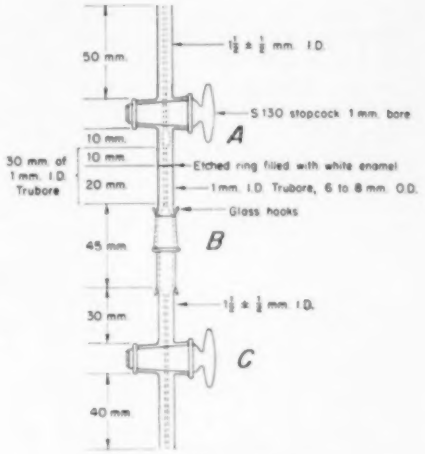

Fig. 1.—Tablet pycnometer.

The *porosity*, or the percentage void space, of a tablet can be determined by evaluating the difference between the specific volume of the tablets and the specific bulk volume of the mixed ingredients. A special tablet pycnometer which was constructed for the determination of the former is illustrated in Fig. 1. The densities of several tablets as determined on the apparatus are given in Table I (1).

The specific bulk volume (the reciprocal of specific density) of the ingredients is best determined on the granulation before compression. A high precision helium densitometer has been designed and constructed for this purpose. Additional, more detailed, information concerning the apparatus, a schematic drawing of which is shown in Fig. 2, will be published elsewhere (2).

Determination of Pore Size.—For a tablet of given porosity, the rate of penetration of liquids into the interior of the tablet is governed by the mean effective pore size. The mean dimension of the pores can be approximated by the following equation:

$$D = V/k \cdot A$$

where

D = the mean effective pore diameter,
A = the specific surface area of the tableted mass in square centimeters per cc.,
k = a geometric constant equal to 3 if the pores are considered spherical in shape,
V = the void space expressed in cc./cc.

Determination of V has already been discussed in the preceding section. A can be determined by the nitrogen gas adsorption method of Brunauer, Emmett, and Teller as reported in a previous publication (3).

Determination of Modulus and Tensile Strength.—The resistance of tablets to mechanical wear as reflected in breakage and abrasion is governed to a large extent by the modulus and tensile strength of the formed tablets. Because of the

TABLE I.—TABLET DENSITY, VOLUME, AND VOID SPACE

	NaBr	NaCl	NaI
Av. wt. (each tablet)	0.2626 Gm.	0.2295 Gm.	0.3439 Gm.
Av. tablet density	3.138	2.119	3.571
Bulk density constituent	3.203	2.165	3.667
Av. tablet volume	0.0836 ml.	0.1083 ml.	0.0963 ml.
Volume of substance	0.0820 ml.	0.1060 ml.	0.0938 ml.
% Void space	1.9	2.1	2.6

present meager state of knowledge regarding these important characteristics of tablets, however, nothing definite can be said at the present.

At the moment no apparatus is available for demining the modulus of tablets. An instrument for this purpose is presently under design. The Strong-Cobb Tablet Hardness Tester is essentially a compressive tensile strength tester and, with some modification, can be used for the purpose.

DYNAMIC FACTORS AND ENERGETICS

The physical process that constituent particles of tablet granulation undergo during the compressional formation of the tablets has received scant attention up to the present time. Basic studies of the physical changes involved, together with their causative forces, nevertheless, are essential if the over-all problem of tablet formulation is to be approached in an intelligent manner.

The compressional process, being essentially nonreversible, is extremely complex. For its partial elucidation a study has been made of the compressional pressure as a function of the degree of compression achieved for a number of pharmaceutical powders and crystals under conditions simulating tablet formation.

Compressional Characteristics of Some Pharmaceuticals at Low Speed.—The literature discloses no reference to studies of pressure-volume type on pharmaceutical powders. The work of Bridgeman (4) on compressibility of inorganic and organic compounds is not concerned with forced reduction in void space, as in the present instance, but rather with reversible compressive increase in the bulk densities of the materials studied.

For procurement of the desired information for this study, the apparatus, a modified $^{3}/_{8}$-in. die and punch set, as shown in Fig. 3, was employed together with a mechanical lever machine shown in Fig. 4. The equipment permitted direct evaulation of the magnitude of the force necessary to produce varying degrees of compression at rates of compression many times slower than those of tableting

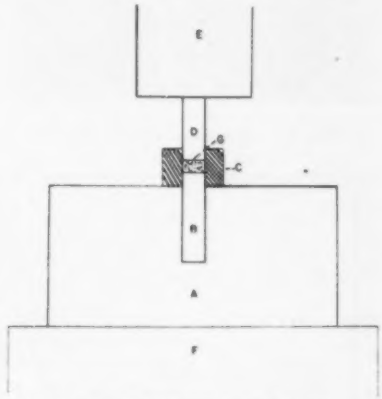

Fig. 3.—Special punch and die set.

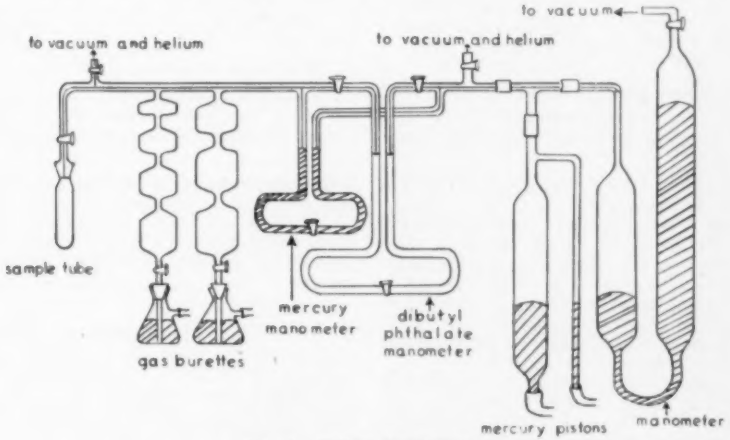

Fig. 2.—Helium densitometer.

Fig. 4.—Mechanical lever machine.

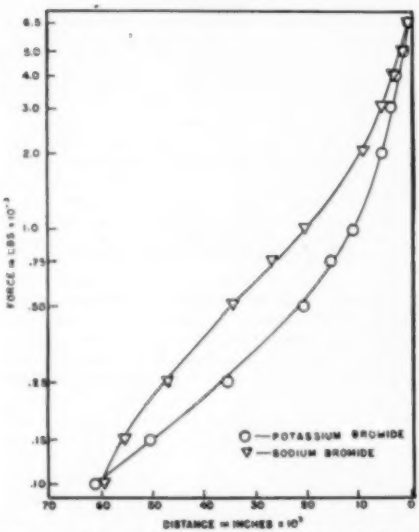

Fig. 5.—Compression curves for some alkali halides.

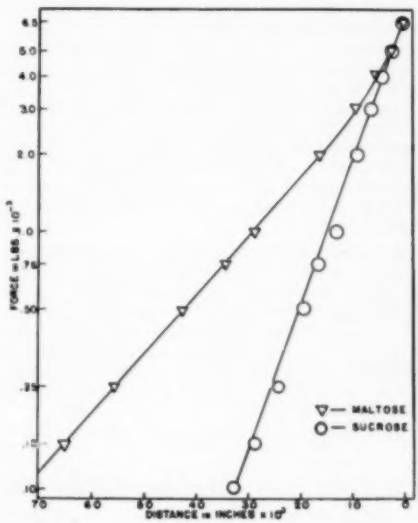

Fig. 6.—Compression curve for some sugars.

machines. More detailed treatment of the method will be given elsewhere (5).

The type of data obtainable is shown in Figs. 5 and 6, where the compression curves of alkali halides and two sugars are given. The curves are quite reproducible for most substances from lot to lot, from operator to operator, and at different times. Usually the extent of compression for a given substance under a given force can be duplicated within a few milliinches.

The significance of two parts of the curves in Figs. 4 and 5 may be discussed at this time. The part of the curve between 1,500 and 2,500 lb. corresponding to pressures of 15,000 to 25,000 lb. seems to be the range of maximum pressure necessary for the formation of pharmaceutically acceptable tablets. At these pressures there is approximately 2–10% void space remaining in the tablet. At maximum pressures studied no measurable void space remains in the case of the halides, and the density of the tablet equals the bulk density of the material composing the granulation. Other substances, such as carbohydrates, still contain an appreciable amount of void space at the maximum pressures used.

Tentative correlations have been achieved between the shapes of the compressional curves and ionic structure, melting point, and hardness of the compressed substances.

The mechanical work of compression can be determined from the data obtained by plotting the compressional force as a function of displacement and determining the area under the curve. This energy should be equal to the sum of the energy dissipated in form of thermal energy and that retained in the tablet in form of increased surface energy. Because of the apparatus used, it is not yet possible to make an energy balance since the thermal energy is lost during the slow compressional process.

Instrumentation of a Tableting Machine.—A fully instrumented tableting machine has been designed and its construction is anticipated. Such an instrument will permit data-taking under dynamic conditions in existence in an actual manufacturing process.

REFERENCES

(1) Higuchi, T., Tucker, S. J., and Busse, L. W., to be published.
(2) Higuchi, T., to be published.
(3) Swintosky, J. V., Riegelman, S., Higuchi, T., and Busse, L. W., THIS JOURNAL, **38**, 210(1940).
(4) Bridgeman, P. W., *Proc. Am. Acad. Arts Sci.*, **70**, 425 (1943).
(5) Arnold, R. D., *et al.*, to be published.

A Spectrophotometric Assay for Diethylstilbestrol and Related Estrogens*

By CLARK A. KELLY† and ARTHUR E. JAMES‡

A colorimetric method involving coupling with diazotized *m*-nitroaniline has been presented whereby it is possible to assay certain pharmaceutical products containing diethylstilbestrol or related synthetic estrogens by comparison with suitable reference standards.

DIETHYLSTILBESTROL and related estrogens may be converted to a highly colored compound by coupling with diazotized *m*-nitroaniline. Photometric determination of this color and that produced by reference standards taken between 500 and 510 mμ, depending on the particular estrogen, constitutes a convenient assay for these estrogens in pharmaceutical products.

Since the introduction of synthetic estrogens by Dodds and his co-workers (1) in 1938, diethylstilbestrol has become official in the U. S P., while Benzestrol, Hexestrol, Mestilbol, and Dienestrol are in the N. N. R. It is the purpose of this communication to show a sensitive and specific yet simple and rapid determination for these synthetic estrogens.

The presence of the phenolic group makes possible a variety of colorimetric procedures (2–8) of which the U. S. P. utilizes the Folin-Denis reagent for the assay of diethylstilbestrol in capsules, tablets, and injections. This method is sensitive but is nonspecific in that the phosphomolybdotungstate complex is reduced by other than phenolic groups. The color so developed exhibits no absorption maximum in the visible range (Fig. 1) but at a selected wave length (600 mμ) the blue color does show good relationship between concentration and percentage transmittancy (Beer's law). The proposed method, however, does have an absorption maximum (Fig. 1) which is used as the wave length for preparing standard graphs. By comparison with these graphs, pharmaceutical products containing the respective estrogens can readily be assayed by measuring directly the percentage transmittancy and reading concentrations from the graph.

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† Fellow of the American Foundation for Pharmaceutical Education. This paper is part of a thesis presented to the Graduate Committee of the Temple University School of Pharmacy by Clark A. Kelly in partial fulfillment of the requirements for the degree of Master of Science.
‡ Professor of Inorganic Chemistry, Temple University School of Pharmacy.

EXPERIMENTAL

Reagents

Standard Diethylstilbestrol Solution.—Dissolve exactly 50 mg. of pure diethylstilbestrol in sufficient amount of 50% ethyl alcohol to make exactly 50 cc. By appropriate dilution with 50% ethyl alcohol prepare a working standard (20 μg./cc.).

Sodium Borate Solution.—Dissolve 5 Gm. in enough distilled water to make 100 cc.

Diazotization Solution.—Dissolve 50 mg. of *m*-nitroaniline in 2 cc. of dilute hydrochloric acid (6 *N*) by warming in a 100-cc. volumetric flask. Chill in ice and add 1 cc. of 5% w/v sodium nitrite solution. After two minutes add approximately 95 cc. of cold distilled water and 1 cc. of 5% w/v sulfamic acid solution. Mix and make to the 100-cc. mark with cold distilled water. Keep in ice bath until ready to use. Make fresh each day.

Sodium Hydroxide Solution.—Dissolve 11 Gm. in enough distilled water to make 100 cc.

Alcohol Solution.—Prepare by mixing equal volumes of absolute alcohol and distilled water.

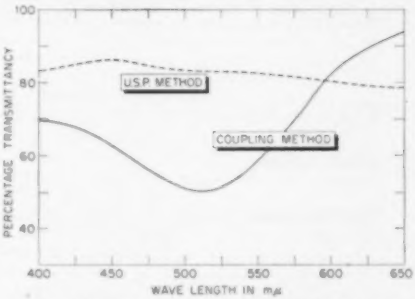


Fig. 1—Absorption spectra of diethylstilbestrol.

Preparation of Standard Graph

To five test tubes graduated accurately at the 10-cc level, add respectively 0, 1, 2, 3, and 4 cc. of the working standard (20–80 μg. of diethylstilbestrol). Dilute each tube with 50% alcohol to give a volume of 5 cc. Add 1 cc. of the 5% sodium borate and then 2 cc. of the cold diazotization solution to each tube. Mix and let stand at room temperature for at least ten minutes. Then add 1 cc. of 10% sodium hydroxide and dilute with distilled water to the 10-cc. mark. Mix well and read the transmittancy of the clear reddish color at an appropriate wave length (510 mμ) after the instrument has been set at 100% transmittancy using the reagent blank solution.[1] Plot these values on semilogarithmic paper against concentration in micrograms. This should give a straight line as shown in Fig. 2.

[1] A Coleman Universal Spectrophotometer Model 14 was used throughout this work.

TABLE I.—ASSAY RESULTS OF DIETHYLSTILBESTROL TABLETS

Tablet Samples	Concentration, Mg.	Coupling Method			U. S. P. Method		
		Found (γ./10 cc.)	Theory	% Error	Found (γ./100 cc.)	Theory	% Error
A	0.1	20	20	0.0	..	..	..
		41	40	2.5	..	..	..
B	0.1	20	20	0.0	..	..	..
		40	40	0.0	..	..	..
C	0.1	21	20	5.0	..	..	..
		40	40	0.0	..	..	..
D	0.25	24	25	4.0	210	200	5.0
		51	50	2.0	..	..	..
E	0.5	20	20	0.0	193	200	3.5
		43	40	7.5	200	200	0.0
F	0.5	21	20	5.0	..	..	..
		39	40	2.5	..	..	..
G	0.5	20	20	0.0	210	200	5.0
		42	40	5.0	..	..	..
H	1.0	42	40	5.0	210	200	5.0
		83	80	3.8	210	200	5.0
I	1.0	42	40	4.0	..	..	..
		79	80	1.2	..	..	..
J	1.0	40	40	0.0	190	200	5.0
		62	60	3.3	..	..	..
K	5.0	50	50	0.0	..	..	..
		48	50	4.0	..	..	..
L	5.0	24	25	4.0	..	..	..
		48	50	4.0	..	..	..
M	5.0	24	25	4.0	210	200	5.0
		48	50	4.0	..	..	..

[a] The authors are greatly indebted to the following companies for their generous supplies of pharmaceutical products used in this study: Abbott Laboratories, American Pharmaceutical Co., Carroll Dunham Smith Pharmacal Co., Central Pharmacal Co., Cole Chemical Co., Endo Products, Inc., Estro Chemical Co., Gold Leaf Pharmacal Co., Kremers-Urban Co., Lederle Laboratories, Eli Lilly and Co., S. E. Massengill Co., Wm. S. Merrell Co., E. S. Miller Laboratories, Premo Pharmaceutical Co., William H. Rorer, Inc., Schieffelin and Co., Sterling-Winthrop Research Institute, Upjohn Co., Vale Chemical Co., Wallace and Tiernan Products, Warren-Teed Products Co., and Winthrop-Stearns Inc.

TABLE II.—SUMMARY OF FIVE RELATED ESTROGENS

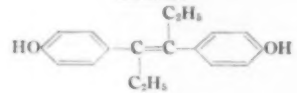
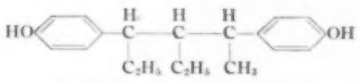
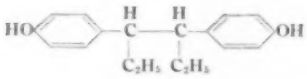
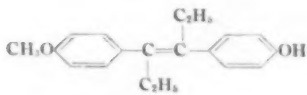
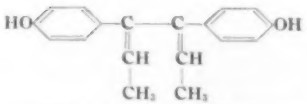
Coupled Estrogen	Structure	Maximum Absorption in mμ	Limits for Beer's Law in γ./10 cc.
Diethylstilbestrol		510	0–80
Benzestrol		500	0–100
Hexestrol		505	0–75
Mestilbol		505	0–100
Dienestrol		500	0–100

TABLE III.—ASSAY RESULTS OF VARIOUS TABLETS BY COUPLING METHOD

Tablet Sample	Concn. in Tablets	μg./10 cc. Found	μg./10 cc. Theory	% Error
N	5.0 mg. of diethylstilbestrol (enteric coated)	20	20	0.0
		42	40	5.0
		19	20	5.0
		40	40	0.0
O	25.0 mg. of diethylstilbestrol (enteric coated)	20	20	0.0
		41	40	2.5
		20	20	0.0
		42	40	5.0
P	2.0 mg. of Benzestrol	25	25	0.0
		50	50	0.0
		50	50	0.0
		100	100	0.0
Q	3.0 mg. of Hexestrol	51	50	2.0
		51	50	2.0
		52	50	4.0
		52	50	4.0

Assay of Diethylstilbestrol Tablets

Weigh 20 tablets and calculate the average weight of a single tablet. Powder an appropriate number of tablets and weigh a sample containing 1–4 mg. of diethylstilbestrol. Transfer to a 50-cc. volumetric flask and add 25 cc. of absolute alcohol. Place on a steam bath for fifteen minutes with occasional swirling. Cool and dilute with distilled water to the 50-cc. mark. Filter the solution discarding the first 10 cc. of filtrate. From the clear filtrate accurately measured aliquots from 0.5 to 4.0 cc. containing 20–80 μg. of diethylstilbestrol, are transferred to the 10-cc. graduated test tubes. These volumes are made up to 5 cc. with the addition of 50% alcohol. The development of color is the same as already described in the preparation of the standard graph. The concentrations of diethylstilbestrol are then determined by reference to the standard graph which is run concurrently.

A few of the results of this assay are given along with the U. S. P. colorimetric method in Table I.

The data in Table I for both methods show values well within the ±10% tolerance allowed by the U. S. P. for diethylstilbestrol.

Assay of Other Pharmaceutical Forms of Diethylstilbestrol

With suitable preparation there is no apparent reason why enteric coated tablets (Table III), ampuls, and aqueous isotonic suspensions of diethylstilbestrol could not be analyzed by this coupling method.

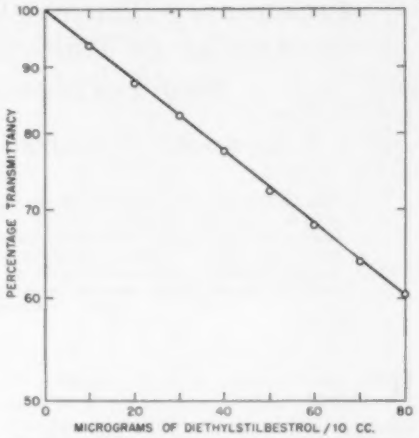


Fig. 2.—Relationship between concentrations of diethylstilbestrol and percentage transmittancy of coupled diethylstilbestrol.

DISCUSSION

The coupling method described herewith is the culmination of preliminary studies in which optimum conditions were established for the maximum intensity and stability of color. Major variables studied were diazotization agents, solvent for diethylstilbestrol, degree of alkalinity and time involved, and concentrations of diethylstilbestrol that adhere to Beer's law at the wave length of maximum absorption. The color so developed is stable for at least twenty-four hours.

By applying the same general procedure the results of the five related pure estrogens are tabulated in Table II.

Results of this coupling method applied to the assay of tablets of the various estrogens which were available are shown in Table III, which again emphasizes the validity of this proposed method.

REFERENCES

(1) Dodds, E., Goldberg, L., Lawson, W., and Robinson, R., *Nature*, **141**, 247(1938).
(2) Dingemanse, F., *ibid.*, **145**, 825(1940).
(3) Decherne, E., THIS JOURNAL, **30**, 208(1941).
(4) Huf, E., and Widmann, G., *Z. physiol. Chem.*, **274**, 88 (1942).
(5) Tubis, M., and Bloom, A., *Ind. Eng. Chem., Anal. Ed.*, **14**, 309(1942).
(6) Cocking, T., *Analyst*, **68**, 144(1943).
(7) Malpress, F., *Biochem. J.*, **39**, 95(1945).
(8) Gottlieb, S., THIS JOURNAL, **36**, 379(1947).

Preliminary Pharmacological Studies of Some Amides of Chloral and *Alpha, Alpha, Beta*-Trichlorobutyraldehyde*,†

By WOODROW R. BYRUM and JOSEPH P. LaROCCA

The HD_{50} and LD_{50} for 19 amide derivatives of chloral and α,α,β-trichlorobutyraldehyde have been determined. Of these compounds, chloral butyramide, chloral isovaleramide, chloral 2-ethylbutyramide, and chloral 2-ethylcaproamide were found to be most active. These compounds compare favorably with chloral hydrate as to milligram potency, therapeutic ratio, and duration of action. In general, the α,α,β-trichlorobutyraldehyde amides were found to be less active than the chloral derivatives.

RECENTLY LaRocca, *et al.* (1), reported the synthesis and fungicidal activity of some chloral and α,α,β-trichlorobutyraldehyde amides. These compounds were made by condensing chloral and α,α,β-trichlorobutyraldehyde with various aliphatic amides. The use of chloral as a hypnotic dates back to 1869 when it was introduced by Liebreich (2). Since then chloral has been largely replaced as a sedative-hypnotic by newer chemical agents. This replacement is perhaps justified in view of disadvantages in its use such as unpleasant taste, irritation to the stomach, large therapeutic doses which sometimes produce dangerous cardiac and respiratory depression, and undesirable physical properties (2). Nevertheless, chloral has distinct advantages such as: prompt and reliable action of adequate duration, easily increased by larger doses; it is readily soluble and its action is noncumulative. α,α,β-Trichlorobutyraldehyde was also introduced as a sedative-hypnotic, but has never acquired extensive use. The aliphatic acid amides have long been used as sedative-hypnotics; for example, ethyl carbamate and carbromal.

In view of these facts, it was thought that the chloral and α,α,β-trichlorobutyraldehyde amides should be investigated for a possible sedative-hypnotic action. Nineteen of these compounds are evaluated in a preliminary way for hypnotic activity and toxic effects. Of these condensation products, only chloral amide has been investigated for hypnotic activity (2). This compound was found to be less active than chloral and its action was attributed to the possible decomposition of the substance to chloral which then exerted its normal action.

The rat was used in this investigation because it has generally been regarded as the most suitable animal for such studies (3). Since the intraperitoneal route of administration has been stated to be the most sensitive method of estimating hypnotic activity in a group comprising compounds of widely different solubilities (3), it was the method of administration used in this study. Fitch and Tatum (4) have suggested the intraperitoneal route as closely approximating slow intravenous injection.

EXPERIMENTAL

Acute Toxicity and Hypnotic Activity.—The acute toxicity and hypnotic activity of 11 chloral amides (Table I) and eight α,α,β-trichlorobutyraldehyde amides (Table II), after single administration, were determined employing the intraperitoneal route in female albino rats weighing 125–175 Gm. Chloral hydrate was injected in parallel as a reference standard. In order to avoid the development of tolerance for the compounds tested, no animal received more than one injection.

All compounds were injected as 5% suspensions in a vehicle consisting of 5% acacia, 10% cottonseed oil, 1% Tween 80,[1] and water. After being homogenized several times, an emulsion was obtained from which the suspended particles of the compound would not readily settle out. Tween 80 has been reported to have an LD_{50} intraperitoneally for the white rat between 8 and 9 cc./Kg. of body weight (5). In this investigation, Tween 80 was found to have no effect in the small concentration used as demonstrated by its lack of effect when injected into control rats in quantities 5 to 10 times that administered during our studies. Our determinations of 472 mg./Kg. for the LD_{50} and 226 mg./Kg. for the HD_{50} for chloral hydrate dissolved in this vehicle (Table I) are in agreement with published reports of 500 mg./Kg. and 200 mg./Kg. for the LD_{50} and HD_{50}, respectively (6). The results listed in Tables I and II, therefore, apparently are not influenced by the solvent.

All suspensions for injection were prepared immediately before using in order to avoid any de-

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Presented to the Scientific Section, A. PH. A., Buffalo meeting, August, 1951.

† This work was supported by a research grant from the University Center in Georgia, Atlanta.

[1] Atlas Powder Company, Wilmington, Del.

TABLE I.—THE ACUTE TOXICITY AND HYPNOTIC ACTIVITY OF CHLORAL AMIDES[a] ADMINISTERED INTRAPERITONEALLY IN RATS

Amides	Total No. Animals	LD_{50} Mg./Kg. and Range	HD_{50} Mg./Kg. and Range	Therapeutic Index, LD_{50}/HD_{50}	Av. Duration Narcosis with HD_{50}, Min.
Acetamide	30	>1200	1015 (957–1090)	>1.18	67
Propionamide	30	>1200	855 (607–1042)	>1.4	65
Butyramide	47	1102 (1023–1190)	389 (327–457)	2.8	60
Isovaleramide	42	>1200	275 (239–306)	>4.15	68
Caproamide	10	Convulsant	Convulsant	..	..
2-Ethylbutyramide	44	965 (790–1115)	490 (454–531)	2.35	26
Oenanthamide	10	>1200	>1200	..	..
Caprylamide	10	>1200	>1200	..	..
2-Ethylcaproamide	62	952 (876–1002)	275 (221–329)	3.46	63
Pelargonamide	30	1026 (974–1087)	978 (908–1058)	1.04	90
Capramide	10	>1200	>1200	..	..
Chloral Hydrate[b]	30	472 (441–510)	226 (212–231)	2.08	37.4

[a] General formula: $RCONHCH(OH)CCl_3$. The *Chem. Abstr.* name for these compounds, N-(2-trichloro-1-hydroxyethyl)-amides, was not used in this paper because it was unwieldy.
[b] Chloral hydrate is included because it was injected in parallel as a reference standard.

composition of the compounds. The time of injection was noted and the rats were observed closely for the onset and duration of hypnotic activity during the first three hours. Symptoms manifested by the animals following the administration of the active compounds were principally those of continuous central nervous system depression. The induction of hypnosis was smooth, except for chloral caproamide, which acted as a convulsant, and recovery from hypnosis was uneventful. The onset of hypnotic effects from the active compounds developed within ten to fifteen minutes after administration for all compounds except chloral propionamide and α,α,β-trichlorobutyraldehyde acetamide. The onset of hypnosis for these two compounds was approximately thirty minutes after injection. The duration of hypnosis was the longest for α,α,β-trichlorobutyraldehyde acetamide, averaging three hundred and sixty minutes. All compounds except chloral 2-ethylbutyramide demonstrated, under these conditions, longer duration of action than the reference, chloral hydrate. Those animals receiving lethal doses died from respiratory paralysis. All deaths occurring within seven days after injection were recorded. The greatest incidence of deaths of the rats occurred in the period between the twenty-fourth and forty-eighth hour following dosage. Only a few deaths occurred after this period. Gross pathologic changes were not observed when surviving animals were sacrificed thirty days after receiving the injections.

The HD_{50} is defined as the dose causing hypnotic effects in 50% of the animals treated. The occurrence of hypnosis was evaluated in terms of the conditions set forth by Nelson, *et al.* (3), and Warren, *et al.* (7). The LD_{50} is defined as the dose killing 50% of the injected animals. The therapeutic index is calculated from the ratio of LD_{50}/HD_{50}. Tables I and II give the results obtained as calculated by the method of Litchfield and Wilcoxon (8). Those hypnotics showing the most promise were evaluated using a minimum of 10 rats at each dosage level. The total number of rats used for each compound is listed in Tables I and II.

DISCUSSION

The results listed in Table I indicate that four derivatives of chloral, the butyramide, isovaleramide, 2-ethylbutyramide and 2-ethylcaproamide have margins of safety which are greater than that for chloral hydrate. The difference is not considerable for the butyramides, but is significant for the isovaleramide and 2-ethylcaproamide. All of these four compounds have a lower milligram potency as hypnotics for the intraperitoneal route in rats than chloral hydrate, but the 2-ethylcaproamide and the isovaleramide are only slightly less potent. This seems to disprove the suggestion by Sollmann (2) that the activity of these compounds is due to a decomposition to chloral or, in the case of the butyl derivatives, to α,α,β-trichlorobutyraldehyde. Upon

TABLE II.—THE ACUTE TOXICITY AND HYPNOTIC ACTIVITY OF α,α,β-TRICHLOROBUTYRALDEHYDE AMIDES[a] ADMINISTERED INTRAPERITONEALLY IN RATS

Amide	No. Animals	Total LD_{50} Mg./Kg. and Range	HD_{50} Mg./Kg. and Range	Therapeutic Index, LD_{50}/HD_{50}	Av. Duration Narcosis with HD_{50}, Min.
Acetamide	34	>1200	670 (572–781)	>1.70	360
Propionamide	42	>1200	390 (318–470)	>3.10	52
Butyramide	42	>1200	416 (349–494)	>2.88	47
Caproamide	10	>1200	>1200		
Oenanthamide	10	>1200	>1200		
Caprylamide	10	>1200	>1200		
Pelargonamide	10	>1200	>1200		
Capramide	10	>1200	>1200		

[a] General formula: $RCONHCH(OH)CCl_2CHClCH_3$. The *Chem. Abstr.* name for these compounds, N-(2,2,3-trichloro-1-hydroxybutyl)amides, was not used in this paper because it was unwieldy.

examining the data for chloral 2-ethylcaproamide and chloral isovaleramide (Table I), it is found that the HD_{50} of each of these compounds upon hydrolysis would yield only a fraction of the HD_{50} for chloral. Further examination of the data shows that the activity of chloral acetamide and chloral propionamide, is less than the activity that would be found if the compounds were hydrolyzed completely to chloral. In the case of chloral propionamide, the HD_{50} of 855 mg. would on hydrolysis yield 571 mg. of chloral which is greater than the LD_{50} for chloral.

A similar analysis of activity can also be made in the case of the α,α,β-trichlorobutyraldehyde amides. A lack of hydrolysis in this group (Table II) is indicated further by the absence of any effects—sedative, hypnotic, or toxic, following the administration of 1,200 mg./Kg. of the caproamide, oenanthamide, caprylamide, pelargonamide, and capramide. In view of these facts, it appears that the sedative-hypnotic effect exhibited by these compounds is a property of the compound and not due to a decomposition product.

In the case of the present study a few generalizations may be made. The chloral amides of Table I show increasing hypnotic activity for the rat as the carbon chain lengthens, with a maximum at seven to eight carbon atoms. Above this point activity falls off very rapidly. The effect of branching of the amide chain appears to enhance the sedative-hypnotic properties; for example, chloral caproamide showed a convulsant action, whereas the 2-ethylbutyramide derivative exhibited a relatively strong sedative-hypnotic action. Even more graphic is the case of chloral caprylamide which was found to be inactive, and that of chloral 2-ethylcaproamide which was found to be the most active compound of those tested.

The α,α,β-trichlorobutyraldehyde derivatives were as a group less active than the chloral derivatives. In this group the peak activity, as in the case of the chloral amides, was found to be in compounds containing seven to eight carbon atoms. For example, the activity of α,α,β-trichlorobutyraldehyde propionamide with seven carbon atoms, does not approximate the activity of the corresponding chloral propionamide, but rather approaches that of chloral butyramide or isovaleramide which contain six and seven carbon atoms, respectively.

Additional studies on the more effective members of the series, involving measurement of sedative effects, LD_{50}, induction time, and duration of sleep following oral administration in rats, and rabbits, is under way at the present time.

SUMMARY

The HD_{50} and LD_{50} for 19 amide derivatives of chloral and α,α,β-trichlorobutyraldehyde have been determined. Of these compounds, chloral butyramide, chloral isovaleramide, chloral 2-ethylbutyramide, and chloral 2-ethylcaproamide were found to be most active. These compounds compare favorably with chloral hydrate as to milligram potency, therapeutic ratio, and duration of action. In general, the α,α,β-trichlorobutyraldehyde amides were found to be less active than the chloral derivatives.

REFERENCES

(1) LaRocca, J. P., Leonard, J. M., and Weaver, W. E., *J. Org. Chem.*, **16**, 47(1951).
(2) Sollmann, T. A., "A Manual of Pharmacology," 7th ed., W. B. Saunders Co., Philadelphia, 1948, p. 673.
(3) Nelson, J. W., Lyster, S. C., and Cartland, G. F., THIS JOURNAL, **30**, 180(1941).
(4) Fitch, R. H., and Tatum, A. L., *J. Pharmacol. Exptl. Therap.*, **44**, 325(1932).
(5) Krantz, J. C., Jr., Culver, P. J., Carr, J., and Jones, C. M., *Bull. School Med., Univ. Maryland*, **36**, 48(1951).
(6) Farris, E. J., and Griffith, J. Q., "The Rat in Laboratory Investigation," 2nd ed., J. B. Lippincott Co., Philadelphia, 1949, p. 332.
(7) Warren, M. R., Thompson, C. R., and Werner, H. W., *J. Pharmacol. Exptl. Therap.*, **96**, 209 (1949).
(8) Litchfield, J. T., Jr., and Wilcoxon, F., *ibid.*, **96**, 99 (1949).

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal, and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Diplococceine
Acetyl strophantin
Adrenostene
3-Amino-*o*-phthalic acid
Biotin sulfone
Diallyl benzophosphate
Galactonic acid
2-Hydroxy-5-mercaptobenzoic acid
2-Isatoxime
Mannonic acid
Oxythiamine
Phosphotyrosine
Piperine
Quinoline-8-carboxylic acid
Sulfosuccinic acid
l-Thiosorbitol
p-Urazine
9-Anthraldehyde
2,6-Diamino-3,7-dimethylacridine
2-Ethylhexanedithiol-1,3
Ethyl propiolate
Ellagic acid

The Simultaneous Spectrophotometric Determination of Gentisic and Salicylic Acids*

By LEWIS J. KLECKNER and ARTHUR OSOL

Aqueous solutions of gentisic acid, adjusted to a *p*H between 5.0 and 8.0, exhibit maximum absorption at 320 mμ, the absorbancy being proportional to concentration at least to 35 mg./L. Solutions of salicylic acid, adjusted within the same *p*H range, exhibit maximum absorption at 296 mμ, the absorbancy likewise being proportional to concentration. At 306.5 mμ, the isobestic point, both gentisic acid and salicylic acid have the same absorbancy in solutions of identical weight concentration. From readings of the absorbancy at 296 and 320 mμ of an aqueous solution containing gentisic and salicylic acids, adjusted to a *p*H between 5.0 and 8.0, the amount of each acid present in the solution may be calculated by means of simultaneous equations. The determination of the absorbancy at 306.5 mμ may be used to confirm the total amount of acids present.

THE IMPORTANT ROLE of gentisic acid as a biological oxidation product of salicylic acid, along with a trend toward prescribing therapeutic combinations containing both gentisate and salicylate, make it desirable to have available a rapid analytical method for the quantitative estimation of the two acids when present in the same sample.

Kapp and Coburn (1), in a study of urinary metabolites of sodium salicylate, separated gentisic acid from salicylic acid by boiling the urine sample with hydrochloric acid and successively extracting with petroleum ether and ethyl ether; the salicylic acid was obtained in the petroleum ether extract, while the gentisic acid was separated from the ethyl ether fraction. Following such separation, a choice of methods may be made for the individual determination of the acids; the gentisic acid, for example, may be estimated by bromination, by reduction of alkaline cupric solution (1), by reaction with a molybdophosphotungstate solution in the presence of alkali (2), or by measurement of the absorbancy of its aqueous solution at 320 mμ (1, 2).

The observation of Osol and Kleckner (3) that gentisic acid produces with molybdophosphotungstate T. S. (U. S. P. XIV) a green color in acid solution, while salicylic acid requires alkalinization for development of color, has made it possible to determine the amount of gentisic acid present in a sample also containing salicylic acid without having to separate the acids.

As gentisic and salicylic acid solutions exhibit maximum absorption in the ultraviolet region at different wave lengths, it appeared likely that simultaneous spectrophotometric determination of the acids might be accomplished. This paper reports on the investigation of this problem and describes the method which was developed and successfully applied to the analysis of mixtures of the two acids.

EXPERIMENTAL AND RESULTS

Apparatus.—All measurements of absorption were made in a Beckman Model DU Spectrophotometer equipped with a hydrogen lamp, using 1-cm. quartz cells to hold the solution and the solvent blank.

Study of Absorption Characteristics.—Aqueous solutions of a given constant concentration of gentisic acid have been shown by us (3) to have constant spectrophotometric absorption characteristics over the range of *p*H 5.0 to 8.0, with maximum absorbancy occurring at 320 mμ. Solutions of salicylic acid of fixed concentration likewise exhibit constant absorption characteristics over the same *p*H range, maximum absorbancy in this case being observed at 296 mμ. Figure 1 shows the separate absorption curves for the two acids, each at a concentration of 25 mg./L. Also shown in the same figure is the absorption curve for a mixture of equal parts of both acids, at a total concentration of 25 mg./L. It may be seen that each point on the curve representing the mixture is the average of the absorbancies of the individual components, i. e., the total absorbancy is the sum of the absorbancies of gentisic and salicylic acids, each at a concentration (in this case) of 12.5 mg./L. At 306.5 mμ both gentisic acid and salicylic acid have the same absorbancy, on an equal weight basis; this wave length will be referred to as the *isobestic* wave length.

The three wave lengths, 296 mμ, 320 mμ, and 306.5 mμ, were selected as the points at which absorption measurements on "known" mixtures of gentisic and salicylic acids were to be made in developing the method. First it was established that the absorbancy of the individual acids was linearly proportional to concentration at each of the selected wave lengths; the concentration range investigated was 15 to 35 mg./L. From these measurements the average absorbancy of each acid at the several wave lengths was calculated for a concentration of 1 mg./L. These values, which are required to set up the equa-

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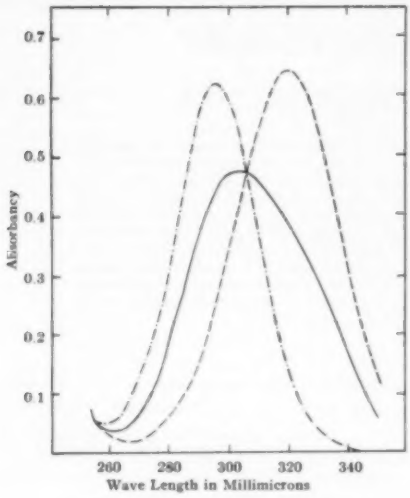

Fig. 1.—Absorption spectra.

——— salicylic acid.
— — — gentisic acid.
——— mixture of acids.

tions for calculating the desired results, are recorded in Table I.

It may be noted that in developing a method for the simultaneous spectrophotometric determination of two components, absorbancy measurements at two different wave lengths (usually at the wave lengths corresponding to maximum absorbancy of the respective components) only are required to provide data for solving the two equations necessary for calculation of the results. Thus, absorbancy data at 296 mμ and 320 mμ would be sufficient for the purpose. The sole reason for determining the absorbancy at the isobestic wave length is to provide a check result on the total amount of the two components (since at this point both components absorb energy equally), thus minimizing the possibility of the presence of absorbing impurities.

Assuming additivity of absorbancies, which had been demonstrated to be justified, the data of Table I were employed to derive the following equations:

$$GA = 41.8\,A_{320} - 8.5\,A_{296}$$
$$SA = 43.1\,A_{296} - 17.1\,A_{320}$$
$$GA + SA = 52.1\,A_{306.5}$$

where GA is the mg./L. of gentisic acid in the solution used in the spectrophotometric measurement, SA is the mg./L. of salicylic acid in the same solution, and the A terms are the absorbancies at the several wave lengths indicated in the subscript.

Method.—The procedure for preparing the solution for the measurement of spectrophotometric absorbance is relatively flexible. If the sample contains only gentisic and salicylic acids, or salts of these acids, sufficient of the sample to represent from 15 to 35 mg. of total acids is dissolved or diluted with sufficient distilled water to make 1 L. of solution; after adjusting the pH, if necessary, within the range of 5.0 to 8.0 by addition of acid or alkali, as the case may be, the absorbancy at the several wave lengths is determined. Distilled water may be used as the solvent blank. Small amounts of sucrose or lactose in the sample do not significantly alter the absorbance. If the sample contains ether-insoluble ingredients which exhibit appreciable absorption at the wave lengths employed in the assay, an acidified aqueous solution of the sample may be extracted with five or more portions of ethyl ether, the solvent evaporated, and the residue containing the gentisic and salicylic acids dissolved in distilled water, the resulting solution adjusted to the proper pH range, and its absorbancy determined. The reliability of the assay is considered satisfactory if the result of the total acid calculation, based on the absorbancy at 306.5 mμ, is within several tenths of a milligram of the sum of the gentisic acid and salicylic acid determinations as calculated from absorbancies observed at 296 mμ and 320 mμ, respectively. The over-all reproducibility, as observed in replicate analyses of known mixtures, is approximately 1% of the amount of each acid present.

Typical results of the application of this method to the analysis of several "known" mixtures prepared by mixing carefully weighed amounts of pure gentisic acid and salicylic acid are shown in Table II; also included in this table are the results of the analysis of compressed tablets containing sodium gentisate and a mixture of salicylates.

Table I.—Average Absorbancies of Solutions Containing 1 Mg./L. (in 1-Cm. Cell)

Wave Length, mμ	Gentisic Acid	Salicylic Acid
296	0.0103	0.0252
320	0.0260	0.0051
306.5	0.0192	0.0192

SUMMARY

1. The absorption characteristics of aqueous solutions of gentisic and salicylic acids have been investigated, in the ultraviolet region, and the average absorbancies at 296 mμ, 320 mμ, and 306.5 mμ calculated for solutions containing 1 mg./L.

2. Equations have been derived by means of which the concentration of gentisic acid and of

Table II.—Results of Analysis of Mixtures Containing Gentisic and Salicylic Acids

Actual Composition		Found Composition		
Gentisic Acid, Mg.	Salicylic Acid, Mg.	Gentisic Acid, Mg.	Salicylic Acid, Mg.	Total Acids, Mg.
12.5	12.5	12.6	12.5	24.8
17.5	17.5	17.3	17.6	34.9
7.5	17.5	7.6	17.4	25.1
17.5	7.5	17.2	7.4	24.9
10.9[a]	17.5[a]	10.9	17.8	29.0
10.9[a]	17.5[a]	10.7	17.6	28.3

[a] Represents the calculated gentisic and salicylic acid equivalents of samples of two lots of tablets containing sodium gentisate and a mixture of salicylates. In the analysis an acidified solution representing an aliquot of the tablet sample was extracted with ethyl ether, the solvent evaporated and the residue of gentisic acid and salicylic acid dissolved in distilled water, the solution adjusted to a pH of approximately 6 and the absorbancy determined.

salicylic acid may be simultaneously, and quickly, determined from observation of absorbancies of an aqueous solution containing the two acids at 296 mμ, 320 mμ, and 306.5 mμ, which wave lengths represent, respectively, the points of greatest absorption by salicylic acid, of greatest absorption by gentisic acid, and of equal absorption by salicylic and gentisic acids.

REFERENCES

(1) Kapp, E. M., and Coburn, A. F., *J. Biol. Chem.*, **145**, 549(1942).
(2) Smith, M. J. H., *J. Pharm. Pharmacol.*, **2**, 439(1950).
(3) Osol, A., and Kleckner, L. J., THIS JOURNAL, in press.

The Influence of Several Drugs upon the Acute Toxicity of Gold Sodium Thiosulfate*,†

By WOODROW R. BYRUM‡ and J. LEON LICHTIN§

The influence of nine compounds upon the acute toxicity of gold sodium thiosulfate in rats and mice is reported. Of the substances investigated, vitamin B complex has been found to be the most effective antidote. Listed in the order of their effectiveness, the following drugs also significantly lowered acute toxicity: for rats—adrenal cortex extract, inositol, caffeine and sodium benzoate, and mercurophylline; for mice—theobromine and sodium acetate, adrenal cortex extract, caffeine and sodium benzoate, and mercurophylline. Ammonium chloride, biotin, and choline chloride were ineffective as antidotes.

GOLD SALTS have been used in the treatment of progressive rheumatoid arthritis since 1927, and since 1935 have come to be recognized as possessing some value in selected and carefully supervised cases (1). In 1948, Hench, *et al.* (2), stated that rheumatologists with few exceptions report results from the use of gold salts better than those obtained with any other single remedy. The advent of ACTH and cortisone has directed attention away from the gold compounds as therapeutic agents in the treatment of rheumatoid arthritis. However, recent work by Margolis and Caplan (3) indicates that the highest percentage of cases obtaining complete remissions or major degrees of improvement occurred in the groups which had received chrysotherapy either concurrently with, or right up to, the time of institution of therapy with the hormone. This report has revived interest in the use of the gold compounds to prevent relapse which occurs in many instances under long-continued therapy with the hormones alone. It is obvious that if these observations prove significant, the employment of chrysotherapy along with ACTH or cortisone would point to one possible means of using the hormones for only a limited time with possible maintenance of the remission subsequently (3).

In spite of the important position which gold will continue to occupy in the treatment of rheumatoid arthritis, any benefit from its use must be weighed against the risk of serious toxic reactions in some 5 per cent of the patients, and minor or moderate transient toxicities in nearly half of the cases in which it is employed (1).

In view of these considerations, i. e., the increasing importance of gold in the treatment of progressive rheumatoid arthritis and the high percentage of toxic manifestations following this therapy, it appeared worth while to screen a number of compounds in order to determine if one could be found which, administered concurrently with the gold, would lessen significantly these toxic effects. Any difference in dose-mortality results between the animals receiving only the gold salt and those also receiving the compound being tested may indicate its value as an antidote (4, 5). Some studies of this nature have already been made. Carratala (6, 7) investigated the antidotal properties of sodium hyposulfite, calcium gluconate, sodium formaldehyde sulfoxylate, and ascorbic acid, and reported

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† This work was started at the College of Pharmacy, Ohio State University, Columbus, Ohio, and completed in the Pharmaceutical Research Laboratories of the University of Georgia.
‡ Professor of Pharmacology, School of Pharmacy, University of Georgia, Athens.
§ Associate Professor of Pharmacy, Cincinnati College of Pharmacy.

that the latter three were most effective in the treatment of chronic and acute gold intoxication. Melville (4) reported that theophylline-sodium acetate exerted no significant influence on the acute toxicity of gold sodium thiosulfate and sodium gold thiomalate, but that theobromine-sodium acetate reduced the acute toxicity of both of those gold compounds. The antidotal effect was most noticeable on the toxicity of the latter compound. Martin and Thompson (5) reported that the acute toxicity of sodium 4-amino-2-aurothiophenyl carboxylate, Krysalgan, was lowered by methionine, cysteine, and cystine, but was little affected by glycine, calcium glycuronate, or ascorbic acid. It should be noted that dimercaprol (BAL) is used in the treatment of severe reactions, but even with the use of dimercaprol, the development of toxicity necessitates immediate discontinuance of aurotherapy. BAL itself may produce unpleasant side effects (1).

This paper reports the preliminary studies on the effects of caffeine and sodium benzoate, theobromine and sodium acetate, adrenal cortex extract, mercurophylline, ammonium chloride, biotin, vitamin B complex, choline chloride, and inositol upon the acute toxicity of gold sodium thiosulfate injected intravenously in mice and intraperitoneally in rats.

EXPERIMENTAL

Effects of Antidotes on Mice.—White female mice, Wistar strain, weighing between 15 and 22 Gm. were used. The dose of the gold sodium thiosulfate was 2.8 mg./20-Gm. mouse and was administered into one of the dorsal tail veins using a 1-cc. tuberculin syringe with a 1/2-inch 27 gauge needle. The volume of solution injected was 0.38 cc. per 20-Gm. mouse administered in thirty seconds. The dose of 2.8 mg. was selected because it was previously determined that this amount of gold sodium thiosulfate would kill between 75% and 100% of the animals (8). The standard dose of the GST, 2.8 mg. per 20-Gm. mouse, was administered intravenously to a control group of 50 mice. The mice were checked several times a day for fourteen days and all deaths occurring during this time were recorded.

The doses of the antidotes used were those obtained by predetermining well-tolerated doses. Each dose of the antidotes listed in Table I was administered in parallel to a group of 10 control animals. No deaths occurred in these groups during the fourteen-day observation period. The drugs being tested as antidotes were injected intraperitoneally fifteen to thirty minutes before the standard dose of GST was administered intravenously. Fifty mice were used in each group. The doses administered and the dose-mortalities of the mice receiving both the GST and the antidote are given in Table I. All deaths occurring during the fourteen-day observation period were recorded.

Effects of Antidotes on Rats.—The experimental work with rats followed, in general, that with the mice. Female albino rats weighing between 125 and 175 Gm. were used. The LD_{75} for GST by the intraperitoneal route was determined using 42 animals (Table I). The graphical method of Miller and Tainter (10) was used to estimate the LD_{75}. This dose, 78 mg./Kg., was administered intraperitoneally to 20 rats in each group fifteen to thirty minutes after the antidote had been injected intraperitoneally. An observation period of fourteen days was used for all groups. All deaths occurring during this time were recorded. The doses administered and the dose-mortalities of the rats receiving both the GST and the antidote are given in Table I.

TABLE I.—EFFECTS OF ANTIDOTES ON THE ACUTE TOXICITY OF GOLD SODIUM THIOSULFATE

	Mouse				Rat			
Compound	Dose GST, Mg./20 Gm. Adm. I. V.	Dose of Antidote per 20 Gm. Adm. I. P.	Mortality Ratio	% Mortality	Dose GST, Mg./Kg. Adm. I. P.	Dose of Antidote per Kg. Adm. I. P.	Mortality Ratio	% Mortality
Gold sodium thiosulfate control	2.8	...	42/50	84	...	...	...	LD_{75} 78 ± 6.2 mg.
Caffeine and sodium benzoate	2.8	1.0 mg.	29/50	58	78	50 mg.	10/20	50
Theobromine and sodium acetate	2.8	3.5 mg.	19/50	38	78	0.15 Gm.	13/20	65
Adrenal cortex ext.[a]	2.8	0.30 cc.	26/50	52	78	15 cc.	9/20	45
Mercurophylline injection, U. S. P.	2.8	0.01 cc.[b]	29/50	58	78	0.5 cc.	11/20	55
Ammonium chloride	2.8	3.0 mg.	38/50	76	78	0.25 Gm.	16/20	80
Biotin[c]	2.8	1.0 mg.	40/50	80	78	20 mg.	15/20	75
Vitamin B complex[d]	2.8	0.2 cc.	13/50	26	78	12 cc.	2/20	10
Choline chloride	2.8	0.1 mg.	38/50	76	78	4 mg.	14/20	70
Inositol	2.8	10.0 mg.	34/50	68	78	0.5 Gm.	10/20	50

[a] Manufactured by the Upjohn Company.
[b] 0.10 cc. of a 1:10 dilution was used.
[c] Biotin sodium injectable "Roche"; 1 cc. contains 5 mg. of *d*-biotin sodium.
[d] Betasynplex Niphanoid, Winthrop-Stearns Inc.; each 2.0 cc. contained the following amounts of the synthetic factors: thiamine HCl 10 mg., riboflavin sodium-sodium tetraborate 5 mg., pyridoxine HCl 5 mg., calcium pantothenate 5 mg., and niacinamide 50 mg.

DISCUSSION AND RESULTS

The time for the administration of the antidote, twenty minutes before the GST was given, was arbitrarily chosen. The optimum time to administer the antidote is an unsettled question. Martin and Thompson (5) found that the detoxifying action was magnified if given orally two hours before the administration of any of several toxic metallic compounds. Melville (4), using the intramuscular route, found no significant difference in the protective action of the antidote when given either simultaneously with, or immediately after, the toxic compound. Gold sodium thiosulfate was used in this investigation because the dose-mortality results were reproducible without too much variation, and the slow chronic toxicity produced by the drug in mice and rats, as contrasted to the more sudden toxicity produced by other gold compounds, is more closely analogous to the delayed type of symptoms characteristic of gold toxicity in humans. Our observations of the contrasting actions of different gold compounds were previously noted by Savin and Warren (8). Animals within a definite weight range were used because Savin and Warren (8) found that, in proportion to weight, the heavier animals could tolerate less and the lighter ones more of the gold compounds.

The selection of those drugs which act as diuretics was based upon the knowledge that gold is eliminated by the kidneys at a much slower rate than it is injected, so that a large cumulation remains in the system for as long as a year after treatment is discontinued. It appeared of interest, therefore, to determine if diuretics would influence the toxicity of gold compounds. Adrenal cortex extract was selected because apparently there is a physiological relationship between the adrenal cortex and ascorbic acid. The latter has been mentioned in several pharmacological investigations of gold compounds both as an antidote (6, 7), and as part of the physiological changes that gold causes (9).

The other compounds tested were selected because of the essential roles they play in the nutrition of the body. Several of these substances are used for the clinical prophylaxis and treatment of hepatic injury, one of the common sequelae of gold therapy.

An analysis of the results listed in Table I shows that, of the compounds investigated, vitamin B complex was the most effective in lowering the acute toxicity of the gold sodium thiosulfate in rats and mice. In mice, the average mortality of 84% in the controls was decreased to 26% among those receiving the standard dose of GST plus the vitamin B complex. In the rats, an expected mortality of 75% was reduced to 10%. The following drugs, listed in the order of their effectiveness, also significantly lowered the acute toxicity in the rats: adrenal cortex extract, inositol, caffeine and sodium benzoate, and mercurophylline. In the mice the acute toxicity was decreased also by the following drugs listed in the order of their effectiveness: theobromine and sodium acetate, adrenal cortex extract, caffeine and sodium benzoate, and mercurophylline. Ammonium chloride, biotin, and choline chloride were found to have no significant action against the acute toxicity of GST in the rats or mice.

Additional work is planned involving: the administration of multiple doses of GST, as is done in clinical practice, along with several doses of the antidotal substances; and a determination of the effect of the antidotes upon the excretion and storage of the gold. This work should indicate further whether those substances which significantly reduced mortality in these experiments may be used clinically to prevent untoward reactions and to increase the usefulness of gold in the treatment of rheumatoid arthritis.

SUMMARY

The influence of nine compounds upon the acute toxicity of gold sodium thiosulfate in rats and mice is reported. Of the substances investigated, vitamin B complex has been found to be the most effective antidote. Listed in the order of their effectiveness, the following drugs also significantly lowered acute toxicity: for rats—adrenal cortex extract, inositol, caffeine and sodium benzoate, and mercurophylline; for mice—theobromine and sodium acetate, adrenal cortex extract, caffeine and sodium benzoate, and mercurophylline. Ammonium chloride, biotin, and choline chloride were ineffective as antidotes.

REFERENCES

(1) "New and Nonofficial Remedies," J. B. Lippincott Co., Philadelphia, 1951, pp. 422, 423.
(2) Hench, P. S., Bauer, W., Boland, E. W., Crain, D. C., Freyberg, R. H., Graham, W., Holbrook, W. P., Lockie, L. M., McEwen, C., Rosenberg, E. F., and Stecher, R. M., *Ann. Internal Med.*, **28**, 66(1948).
(3) Margolis, H. M., and Caplan, P. S., *J. Am. Med. Assoc.*, **145**, 382(1951).
(4) Melville, K. I., *Revue can. biol.*, **3**, 442(1944).
(5) Martin, G. J., and Thompson, M. R., *J. Exptl. Med. Surg.*, **1**, 38(1943).
(6) Carratala, R., *Rev. argentino-norte. amer. cienc. méd.*, **1**, 480(1943), as reported by *Chem. Abstr.*, **40**, 640(1946).
(7) Carratala, R., *Rev. med. munic (Rio de Janeiro)*, **5**, 375(1943), as reported by *Chem. Abstr.*, **38**, 2744(1944).
(8) Savin, A. B., and Warren, J., *J. Bact.*, **40**, 823(1940).
(9) Zwemer, R. L., and Elftman, H., *J. Lab. Clin. Med.*, **31**, 333(1946).
(10) Miller, L. C., and Tainter, M. L., *Proc. Soc. Exptl. Biol. Med.*, **57**, 261(1944).

The Assay of Ephedrine Colorimetrically in Pharmaceutical Preparations*

By L. G. CHATTEN and L. I. PUGSLEY†

Ephedrine in benzene reacts with picryl chloride to produce a color which is measured at 400 mμ and gives a linear relationship when concentration is plotted against absorbancy between the limits of 0.1 and 0.6 mg. of ephedrine. Procedures are given for the assay of ephedrine and ephedrine hydrochloride in various types of commercial preparations.

SEVERAL METHODS of assay of pharmaceutical preparations containing ephedrine have been reviewed by Welsh (1) and a method was proposed (2) based on extracting the drug from an alkaline solution and determining gravimetrically the amount of ephedrine as the hydrochloride. When this method was applied, by the authors, to ephedrine preparations of oil or syrups, it was found that the amount of drug was overestimated, due to the contamination of the residue with extraneous material.

Colorimetric methods of assay have been described by Allport and Jones (3) and Ulrix (4), who adapted the color reaction of Chen and Kao (5) to a quantitative determination of ephedrine. These methods were investigated by us because they appeared to be simpler and more readily adaptable than any of the other colorimetric methods. They were found to be too insensitive, however, to be satisfactory for quantitative determinations. Although a linear relationship was established over a wide range, to obtain an increase in the intensity of color required large amounts of ephedrine.

EXPERIMENTAL

The reaction of picryl chloride with ephedrine, which is covered by the principle of vinylogy proposed by Fuson (6), was investigated and found to yield a linear relationship between the limits of 0.1 to 0.6 mg. of ephedrine.

The absorption spectrum of the reaction mixture of ephedrine and picryl chloride was determined with a Beckman DU Spectrophotometer. Nine milliliters of benzene containing 0.2 mg. of ephedrine and 1 ml. of 0.3% of picryl chloride was allowed to react for twenty-four hours and the absorbancy of the colored mixture read over the range of 340 to 520 mμ. Readings were taken every 10 mμ except between 395 and 410, where they were taken every 2 mμ with the result that a very sharp peak was demonstrated. It was necessary to determine the absorption spectrum on the relatively stable colored complex obtained after allowing the reaction to proceed for twenty-four hours rather than that obtained under the conditions of the assay, because under the latter conditions the intensity of the color tends to increase after removal from the water bath. The absorption spectrum obtained is plotted in Fig. 1 showing a maximum absorbancy at 400 mμ. On the basis of these results, a filter transmitting in this range was selected for the method (filter 400, Evelyn colorimeter).

Tests were made to select a suitable period of heating for the reaction mixture which would yield a satisfactory sensitivity and differentiate between increments of ephedrine. To colorimeter tubes containing 0.1 and 0.6 mg. of ephedrine in 9 ml. of benzene, respectively, was added 1 ml. of 0.3% picryl chloride. Individual series of five tubes were heated in a water bath at 75–77° for five, ten, fifteen, twenty, twenty-five, and thirty minutes respectively and the galvanometer readings taken. The results are shown in Table I. On the basis of these results a period of heating of twenty minutes was selected as the most convenient and satisfactory for assay purposes. Although the reaction is not complete at the end of this time, a satisfactory degree of sensitivity is evident with a good degree of differentiation between different increments of ephedrine.

PROCEDURE

The following procedure was adopted after considerable experimental work to obtain satisfactory concentrations of reagents.

Reagents

Sulfuric acid, 10% w/v
Ethyl ether, reagent grade
Chloroform, reagent grade
Benzene, reagent grade
Picryl chloride, 0.3% in benzene
Sodium hydroxide, 20%
Sodium carbonate, anhydrous
Sodium chloride
Ephedrine, 0.01% in benzene

Transfer 1, 2, 3, 4, 5, and 6 ml. of 0.01% standard ephedrine solution to each of six colorimeter tubes and adjust the volume of each to 9 ml. with benzene. Add 1 ml. of 0.3% picryl chloride to each tube and to a blank containing 9 ml. of benzene. Beginning with the addition to the first tube and until all the tubes have received their quota of picryl chloride, and have been stoppered, a period of exactly two minutes is allowed. Immediately place the stoppered tubes in a water bath at 75–77° for exactly twenty minutes. At the end of this time remove

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TABLE I.—THE EFFECT OF THE TIME OF HEATING ON THE DEVELOPMENT OF COLOR WITH PICRYL CHLORIDE AND EPHEDRINE

Time of Heating, Min.	Galvanometer Readings Ephedrine, 0.1 Mg.	Ephedrine, 0.6 Mg.	Ratio, 0.1 Mg. / 0.6 Mg.
5	84.50	58.25	1.45
10	81.75	49.75	1.64
15	79.00	35.50	2.22
20	75.00	27.75	2.72
25	71.75	22.75	3.15
30	67.75	18.00	3.71

the tubes from the water bath and after exactly three minutes at room temperature, read in an Evelyn colorimeter using filter 400 with the blank at 100% transmission. Using the above procedure, the relationship between the per cent transmission and amounts of ephedrine varying from 0.1 to 0.6 mg. was determined. The results are shown in Table II. Each point is the mean of 10 determinations.

TABLE II.—THE RELATIONSHIP BETWEEN THE CONCENTRATION OF EPHEDRINE AND PER CENT TRANSMISSION

Ephedrine, Mg./10 ml.	Transmission, %
0.1	75.00 ± 0.50[a]
0.2	60.50 ± 0.50
0.3	49.75 ± 0.50
0.4	41.00 ± 0.37
0.5	33.50 ± 0.50
0.6	27.50 ± 0.42

[a] ± One standard deviation.

The relationship between optical density and amount of ephedrine may be represented by the linear equation $y = 0.0415 + 0.8667\ x$ calculated by the method of least squares, y for optical density and x for mg. of ephedrine.

Methods for Various Types of Preparations

Aqueous Isotonic Sprays and Water-Soluble Jellies.—Measure accurately a quantity of the sample equivalent to 50–100 mg. of ephedrine hydrochloride and transfer to a separatory funnel with approximately 20 ml. of water. Render the solution alkaline to litmus with 20% sodium hydroxide and then add an additional 0.5 ml. of the alkali. Extract the solution with six 15-ml. portions of benzene, combine the benzene extracts in a 100-ml. volumetric flask and make up to volume. Pipette an aliquot of the benzene extract equivalent to 0.4 mg. of ephedrine into each of five Evelyn colorimeter tubes and adjust the volume to 9 ml. with benzene. Proceed with the determination as described above, beginning with "add 1 ml. of 0.3% picryl chloride," etc.

Syrups.—Proceed as with aqueous isotonic sprays. If emulsions occur during the extraction with benzene, the following procedure has been found satisfactory: measure accurately the equivalent of 50–100 mg. of ephedrine hydrochloride and transfer to a separatory funnel. Add 10 ml. of water and make the solution alkaline with 20% sodium hydroxide. Extract the ephedrine from the mixture with six 15-ml. portions of ether or until the ephedrine is completely extracted. Combine the ether

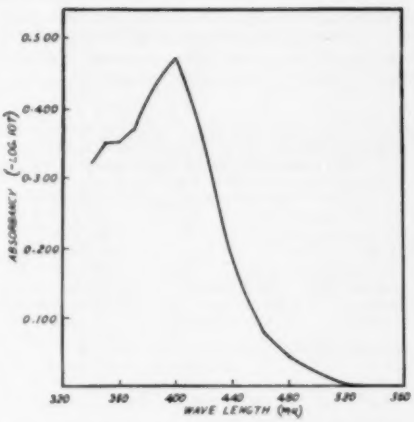


Fig. 1.—Absorption spectrum of colored derivative of ephedrine (0.2 mg. ephedrine in 10 ml.).

solutions and extract first with 5 ml. of 10% sulfuric acid and then with four 5-ml. portions of water. Proceed with the determination as described above beginning with "render the solution alkaline with 20% sodium hydroxide," etc.

Capsules.—Transfer the contents of 20 capsules to a beaker and add 10 ml. of water. Place the empty capsules in another beaker and wash with 10 ml. of water. Filter off the empty capsules and add the solution to the first beaker. Transfer the combined solutions to a separatory funnel and proceed with the determination as described under aqueous isotonic sprays beginning with "render the solution alkaline with 20% sodium hydroxide," etc.

Tablets.—Weigh 20 tablets and powder with a minimum of loss, take an aliquot equivalent to 50–100 mg. of ephedrine hydrochloride, dissolve in 20 ml. of water and filter into a separatory funnel. Wash the filter paper with water and add approximately 500 mg. of anhydrous sodium carbonate and sufficient sodium chloride to form a saturated solution. Proceed with the determination as described above beginning with "extract the solution with six 15-ml. portions of benzene."

Oily Nose Drops.—Measure accurately a quantity which contains 50–100 mg. of ephedrine. Transfer to a separatory funnel and add approximately 10 ml. of ether. Extract with 5 ml. of 10% sulfuric acid and then with four 5-ml. portions of water. Combine the acid and aqueous extracts and render alkaline with 6–7 ml. of 20% sodium hydroxide. Proceed as with aqueous isotonic solutions beginning with "extract with six 15-ml. portions of benzene."

Ointments.—Weigh accurately a quantity of ointment which will contain approximately 10 mg. of ephedrine. Dissolve this sample in ether and filter into a separatory funnel, wash the filter paper with ether and proceed as with oily nose drops beginning with "extract with 5 ml. of 10% sulfuric acid."

In order to determine the accuracy of the method, various types of ephedrine preparations, corre-

sponding to popular commercial formulas were made containing known amounts of the drug. These were assayed according to the procedures described above. The results are shown in Table III.

TABLE III.—THE RECOVERY OF KNOWN AMOUNTS OF EPHEDRINE OR EPHEDRINE HYDROCHLORIDE IN DIFFERENT TYPES OF PREPARATIONS

Type of Preparation	Recovery, %
Oily nose drops (A)	99.7 ± 0.67[a]
Oily nose drops (B)	99.5 ± 0.71
Syrup	100.0 ± 0.44
Aqueous isotonic spray	101.0 ± 0.56
Ointment (A) (simple base)	97.5 ± 0.50
Ointment (B) (simple base)	97.6 ± 0.47
Ointment (C) (soft paraffin base)	98.4 ± 0.61

[a] ± One standard deviation.

It is seen that the recoveries are well within the limits of experimental error.

The colorimetric method has been applied to a number of pharmaceutical preparations and a comparison made with the gravimetric procedure of Welsh (2) in several instances. These results (mean of five determinations) are shown in Table IV. It is seen that the recovery of the labeled amount of the drug tends to be overestimated in most of the preparations using the gravimetric procedure, while the colorimetric procedure gives results more in agreement with the expected value. No explanation other than inadequate mixing on the part of the manufacturer can be given for the low results obtained with the two ointments and some of the oily nose drop preparations.

TABLE IV.—A COMPARISON OF THE RESULTS OBTAINED ON COMMERCIAL PREPARATIONS OF EPHEDRINE BY USING THE COLORIMETRIC METHOD AND THE METHOD OF WELSH

Type of Product	Recovery of Labeled Amount, % Gravimetric	Colorimetric
Oily nose drops		
Product No. 1	...	101.3
2	...	106.5
3	...	84.25
4	...	81.1
5	...	80.0
6	113.7	75.0
7	109.5	97.3
Aqueous isotonic nose drops		
Product No. 1	108.0	93.0
2	112.5	102.8
3	113.5	106.6
4	108.5	105.5
5	110.6	106.5
Water-soluble jelly		
Product No. 1	109.3	107.8
2	97.3	94.5
3	...	93.0
Syrup ephedrine HCl		
Product No. 1	135.1	98.2
2	196.8	102.5
3	...	58.8
4	166.8	100.2
Tablets ephedrine HCl		
Product No. 1	114.7	106.2
2	113.1	104.8
3	...	99.5
Capsules ephedrine HCl		
Product No. 1	101.5	99.8
2	110.0	106.7
Ointment		
Product No. 1	36.1	33.4
2	32.3	22.3

DISCUSSION

Ephedrine, a secondary amine, reacts with picryl chloride to produce a compound which exhibits an intense yellow color. The reaction is not necessarily specific for ephedrine in that other compounds possessing the same chemical groups might yield a similar colored complex. The results appearing in Table II, using definite increments of ephedrine, show that a linear relationship is obtained between 0.1 and 0.6 mg. of ephedrine.

The reaction between the two substances at room temperature tends to be slow and, in order to increase the rate of reaction, heat was applied. The most favorable and applicable time and temperature found for the reaction was 75 to 77° and a heating period of twenty minutes. Even under these conditions the reaction is not completed, hence the necessity of adhering closely to the conditions described. Benzene was selected as a suitable medium for the reaction. Its boiling point is sufficiently high to permit its use in the assay and it is a satisfactory solvent for ephedrine.

A period of two minutes is allowed for the addition of the picryl chloride reagent. This was selected arbitrarily in order to secure uniformity in each case. When the tubes are removed from the water bath, a period of three minutes was chosen. This was also selected arbitrarily in order to allow time for cooling and to permit the drying of the tubes before insertion in the colorimeter. The readings are taken then in rapid succession.

SUMMARY

A colorimetric procedure for the assay of ephedrine preparations has been developed based on the coupling of ephedrine and picryl chloride in benzene solution at 75 to 77°. The application of the method to various types of ephedrine preparations is described.

REFERENCES

(1) Welsh, L. H., *J. Assoc. Offic. Agr. Chemists*, **30**, 467 (1947).
(2) Welsh, L. H., *ibid.*, **31**, 113(1948).
(3) Allport, N., and Jones, N., *Quart. J. Pharm. Pharmacol.*, **15**, 238(1942).
(4) Ulrix, F., *Bull. soc. roy. sci. Liege*, **12**, 312(1943).
(5) Chen, K. K., and Kao, C. H., THIS JOURNAL, **15**, 625 (1926).
(6) Fuson, R. C., *Chem. Revs.*, **16**, 1(1935).

Notes

A Note on the Influence of Caffeine on the Enzyme Urease*

By PHILIP C. MERKER† and RALPH H. CHENEY‡

THE TRIMETHYLXANTHINE, caffeine, has been shown to affect some enzyme systems and not others. Slight retarding effect of caffeine on liver and brain reductase (1); absence of any effect on frog muscle catalase (2); and definite inhibitory action on muscle adenosinetriphosphatase (3); and nerve cholinesterase (4) have been reported in the literature.

Experimental evidence showing the lack of significant effect of caffeine upon the enzyme urease from jack bean meal is herein presented. This work was initiated during a study of the influence of this purine on growth and plant cytology which will be reported later.

EXPERIMENTAL

Urease activity was determined using the Harrow adaptation (5) of the Marshall (6) and Van Slyke and Cullen (7) techniques. A solution containing 32 mg. % urea was used instead of normal blood.

The urease-caffeine preparation was made by mixing, for one minute, 1 Gm. of commercial urease preparation from jack bean meal with 9 cc. of a saturated solution of caffeine, U. S. P., in a glass mortar. The mixture was allowed to stand at room temperature without stirring for fifteen minutes. Then 1 cc. of the preparation was added to an aeration tube containing 3 cc. of a 32 mg. % urea solution and 3 cc. of phosphate buffer *p*H 7.6.

The tube was shaken briefly and the contents were permitted to react for fifteen minutes, after which time 10 cc. of a saturated solution of potassium carbonate and 1 cc. of a 1:4 caprylic alcohol solution were added. Aeration was then initiated and continued for thirty minutes through a standard HCl solution. The residual acid was titrated with standard NaOH. Blank determinations using 3 cc. of distilled water instead of the 32 mg. % urea solution were run simultaneously. All determinations were made at room temperature with freshly made urease-caffeine preparations.

Additional experiments were performed using a buffered phosphate solution of *p*H 6.4 and still others with a centrifuged urease preparation.

The centrifuged preparation employed, in lieu of that described by Jansen (8), was made by mixing 1 Gm. of the commercial urease with 9 cc. of a saturated solution of caffeine, U. S. P. The mixture, after standing at room temperature without stirring for twelve minutes, was centrifuged at high speed for three minutes in a Clay-Adams Jr. A. C. micro centrifuge.

One cubic centimeter of the resulting supernatant fluid was utilized in separate determinations with the phosphate buffers of *p*H 7.6 and 6.4, respectively. Freshly made centrifuged preparations were employed for each series of determinations.

Values obtained are recorded in Table I.

TABLE I.—UREASE ACTIVITY EXPRESSED AS MG. % UREA RECOVERED AS UREA FROM A 32 MG. % UREA SOLUTION

	Uncentrifuged				Centrifuged			
	*p*H 6.4		*p*H 7.6		*p*H 6.4		*p*H 7.6	
	Mg. % Urea	S. E.	Mg. % Urea	S. E.	Mg. % Urea	S. E.	Mg. % Urea	S. E.
Control:	25.2	0.33	26.0	0.41	24.7	0.42	25.3	0.84
Caffeine:	26.0	0.62	28.3	0.51	24.9	0.26	26.1	0.30

CONCLUSION

Urease activity, expressed as mg. per cent urea recovered at *p*H 6.4 and *p*H 7.6, is not affected significantly by a saturated solution of caffeine, U. S. P.

REFERENCES

(1) Harris, D. F., and Creighton, H., *J. Biol. Chem.*, **22**, 535(1915).
(2) Santesson, C. G., *Skand. Arch. Physiol.*, **39**, 132(1920).
(3) Torda, C., and Wolff, H. G., *Am. J. Physiol.*, **152**, 86 (1943).
(4) Nachmansohn, D., and Schneeman, H., *J. Biol. Chem.*, **159**, 239(1945).
(5) Harrow, B., "Laboratory Manual of Biochemistry," 2nd ed., W. B. Saunders Co., Philadelphia, 1948, pp. 70–72.
(6) Marshall, E., *J. Biol. Chem.*, **15**, 487(1913).
(7) Van Slyke, D., and Cullen, G., *ibid.*, **19**, 211(1914); **24**, 117(1916).
(8) Jansen, B. C. P., *Chem. Weekblad*, **12**, 483(1915).

* Received July 6, 1951, from 113 Wiggins St., West Lafayette, Ind.
† Long Island University, Brooklyn College of Pharmacy, Brooklyn, N. Y. Present address: Purdue University, School of Pharmacy, Lafayette, Ind.
‡ Professor of Biology, Brooklyn College, and the Brooklyn College of Pharmacy, Brooklyn, N. Y.

Apparatus for Removal of Light Powder from Large Containers*

By REGINALD F. JOHNSON

MANY diluted solutions of labile biologicals may be concentrated efficiently by the lyophlic process. When large steel containers are used for this process, some difficulty may be experienced when attempting to remove the relatively small amounts of the powder in the can after drying. When the can is inverted, most of the powder is trapped on the shoulders of the can. Also the large cans are too deep to permit removal of the powder by hand with a scoop. The apparatus described below eliminates both of these difficulties, since the pick-up tube can be long enough to reach into the largest cans normally used when lyophilizing large quantities of liquid, and, since the cans need not be be inverted, the shoulder construction is immaterial when the cans are being emptied.

DESCRIPTION

The apparatus operates on the principle of the fluid flow of solid particles in an air stream. It consists of a pick-up tube, receiver, and accessories.

The pick-up tube may be glass or stainless steel, one-half inch inside diameter, extending from the bottom of the can to about 12 inches above the aperture of the can. The receiver should be a wide-mouthed bottle of approximately two-liter capacity, with the appropriately sized stopper. The vacuum line should be about one-fourth inch outside diameter glass tubing extending from above the stopper to about an inch from the bottom of the bottle. The lower end of the tube should be covered with six layers of cheesecloth held over the tube end with a rubber band. This provides a disposable powder retaining air filter. The components should be assembled as in the drawing.

OPERATION

The bottle should be tared, the stopper with tubing inserted, the entire assembly inverted over the can to be emptied, the vacuum line opened and the can swept out with the pick-up line. When the receiving bottle is about three-fourths full, the tubing assembly may be removed and the bottle reweighed.

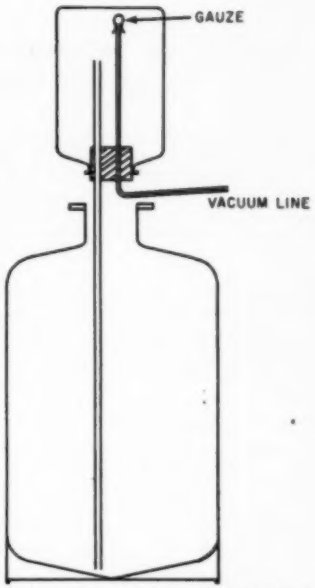


Fig. 1.—Apparatus for Removal of Dry Powder from Large Lyo Cans.

SUMMARY

With this equipment, large volumes of solutions having a very low percentage of solute may be freeze-dried and the solids efficiently recovered.

* Received October 20, 1951, from the Department of Immunochemistry, Research Division, Sharp & Dohme, Inc., Glenolden, Pa.

BOOKS RECEIVED

Aids to Pharmaceutical Chemistry. By WILFRED A. MUTEHAM. Bailliére, Tindall & Cox, London, 1951. 347 pp. 10.3 x 16.3 cm. Price $2.

The Phosphatides. By HAROLD WITTCOFF. Reinhold Publishing Corporation, New York, 1951. xviii + 564 pp. 16 x 23.5 cm. Price $10.

Essentials of Pharmacology. 2nd ed. By FRANCES K. OLDHAM, F. E. KELSEY, and E. M. K. GEILING. J. B. Lippincott Co., Philadelphia, 1951. xvi + 462 pp. 13 x 20 cm. Price $5.

A Study of Antimetabolites. By D. W. WOOLLEY. John Wiley & Sons, Inc., New York, 1952. xiii + 269 pp. 15.5 x 23.5 cm. Price $5.

Pharmacology and Pharmacotherapeutics for Dentists 4th ed. By WILLIAM H. O. MCGEHEE and MELVIN W. GREEN. The Blakiston Company, Philadelphia, 1952. xviii + 550 pp. Illustrated. 16 x 23.5 cm. Price $7.50.

Fluorine and Its Compounds. By R. N. HASZELDINE and A. G. SHARPE. John Wiley & Sons, Inc., New York, 1951. 153 pp. 11 x 17 cm. Price $1.75.

The Lipids: Their Chemistry and Biochemistry. Vol. I. By HARRY J. DEUEL, Jr. Interscience Publishers, Inc., New York, 1951. xxiv + 982 pp. 16.5 x 23.8 cm. Price $18.50.

Book Reviews

Laboratory Guide in Pharmacology for Pharmacy and Dental Students. Revised ed. By HARALD G. O. HOLCK. Burgess Publishing Co., Minneapolis, 1951. xiv + 129 pp. 21 x 27.5 cm. Price $2.75.

In the revised edition of this excellent laboratory guide, the author has retained the many fine features of the original. Wherever possible the experiments have called for the use of the smaller and less expensive animals. Emphasis has been placed on the use of relatively simple experiments which work when in the hands of the student. All too often pharmacological experiments do not work because the surgery and the physiological setup is too difficult for the novitiate. Further, when experiments of this type are used, the emphasis falls on the technique rather than on the scientific principle involved.

New experiments have been added on the use of dimercaprol as an antidote for arsenicals, tolerance and cross-tolerance to Nostal, as well as new material concerning inflammatory edema and trimethadione-pentylenetetrazol antagonism. The elaboration of the section on bioassay is especially helpful and, in this connection, the discussion of the statistics of the digitalis assay is the most lucid explanation that the reviewer has seen that was not intended for statisticians.

Teachers will be pleased that illustrative films available are still listed and in fact re-evaluated so that the more "medical" ones have been deleted.

Those familiar with the first edition of Dr. Holck's book will appreciate the clearer printing and especially the exquisite illustrative drawings.

Particularly those teachers not originally trained in pharmacology will find this laboratory guide valuable because of clarity of direction and experiments that have been time-tested.—MELVIN W. GREEN.

Radioactivity Applied to Chemistry. Edited by ARTHUR C. WAHL and NORMAN A. BONNER. John Wiley & Sons, Inc., New York, 1951. xv + 604 pp. 15.5 x 23.5 cm. Price $7.50.

This book makes available the results of a comprehensive search of the literature in this expanding field. The 12 contributing authors are actively associated with the special subjects upon which they report. They have managed to include a great deal of information with illustrative examples from the literature to give a clear picture of the present knowledge about a very technical subject. However, the presentation is good and the reading is interesting even for those who cannot follow the mathematical developments which are included only where they are necessary for complete presentation.

The book is divided into two parts. The first part consists of 10 chapters of text material describing the nature of the contributions that have been made to various branches of chemistry through the application of radioactivity. The possibilities and the limitations of the various applications are discussed. The second part of the book consists of 18 extensive tables which, under descriptive titles, e. g., Hot-Atom Chemistry—Induced Nuclear Reactions, supply condensed data and literature references. The order, subject matter, and numbers of the tables correspond to those of the related preceding chapters. The text includes summaries and references to applications of radioactivity to chemistry available up to 1950. Some aspects of radiochemistry, i.e., chemical work with radioactive materials, are not summarized. Topics such as radiochemical techniques, the preparation of tagged compounds, and the isolation of radioactive nuclides by use of isotopic carriers are omitted. Applications of stable tracers are not included.

A table of symbols and abbreviations is given at the beginning of the book. The abbreviations are undoubtedly widely used in this field of publication. It would be helpful if authors of books on chemical subjects would use standard abbreviations. The abbreviations used in *Chemical Abstracts* are excellent and have been time-tested. There is no good reason for using "evap." for evaporate, evaporated, and evaporation when only one more letter (*d* or *n*) would be so helpful.

This book was written for the reader with a good knowledge of chemistry, but only an elementary knowledge of radioactivity is essential. It is primarily a reference book, and it is an exceptionally good one, but it can be used, and is being used, as a text for senior undergraduate and graduate courses in applied radiochemistry. There are included 39 pages of bibliography and 28 pages of index.

Wherever research time in this field is valuable this book should be immediately available.

Reagent Chemicals. American Chemical Society Specifications, 1950. American Chemical Society, Washington, D. C., 1951. xiii + 400 pp. 16 x 24.2 cm. Price $5.

This book, compiled by the Committee on Analytical Reagents of the American Chemical Society working during a period of many years, is of special significance to pharmaceutical chemists engaged in analytical research and control. It presents specifications for analytical reagent grade chemicals in which trace impurities are limited to the lowest point consistent with the best chemical manufacturing practice. These specifications, many of which have previously been published in *Analytical Chemistry* and in its predecessor, have for many years, served the United States Pharmacopeia and the National Formulary most effectively as a source of adequate standards for many of the reagent chemicals described in these two official drug compendia. The quality of these standards for reagent chemicals has been assured by the adequacy of the procedures used by the Committee on Analytical Reagents, and the scientific integrity of the members of that committee.

As pointed out by Walter J. Murphy, editor of *Analytical Chemistry*, in his foreword to the book, "The American Chemical Society committee that has labored so long and so conscientiously in preparing this book has numbered among its members chemists from several American producers, who have contributed knowledge and skill that come from production. The membership has always included, also, chemists from laboratories interested only as users of reagents. Some of these laboratories have, for many years, systematically tested reagent chemicals for purity. This pooling of the experience of many laboratories, and the balancing of the points of view of producers and users, give this book a unique value, not shared by those that have preceded it."

As in the U. S. P. and the N. F., the design of each monograph follows as nearly as possible the same pattern. Following the name of the reagent chemical, its molecular formula and the formula weight are given. The requirements stating the purity if an assay is required, and the maximum permitted percentage of trace impurities are outlined. This is followed by the tests applied to determine the requirements outlined.

The revision committees of the U. S. Pharmacopeia and of the National Formulary are deeply indebted to the members of the committee responsible for the preparation of this edition. They have always cooperated most generously in supplying advance information on specifications for inclusion in the U. S. P. and the N. F. As a result, uniformity of standards for reagent grade chemicals has been promoted, and those responsible for U. S. P. and N. F. specifications have been spared much time and effort that otherwise might have been directed toward the problems involved in developing adequate quality and purity specifications for these important analytical necessities.

1952 Iodine Research Award Nominations Requested

Nominations are now being received by the AMERICAN PHARMACEUTICAL ASSOCIATION for the 1952 Chilean Iodine Educational Bureau Award recognizing outstanding research in the chemistry and pharmacy of iodine and its compounds as applied in pharmacy or medicine. Any member of the ASSOCIATION may propose a nominee by submitting eight copies of each of the publications to be considered in the competition, a biographical sketch of the nominee including date of birth, and a list of his publications. Eight copies of the nomination must be submitted to Robert P. Fischelis, Secretary of the AMERICAN PHARMACEUTICAL ASSOCIATION, 2215 Constitution Ave., N. W., Washington 7, D. C. To be eligible for the 1952 Award, nominations must be received on or before August 1, 1952.

A nominee must be a resident of the United States or Canada. He must have accomplished outstanding research in the chemistry or pharmacy of iodine and its compounds as applied in pharmacy or medicine.

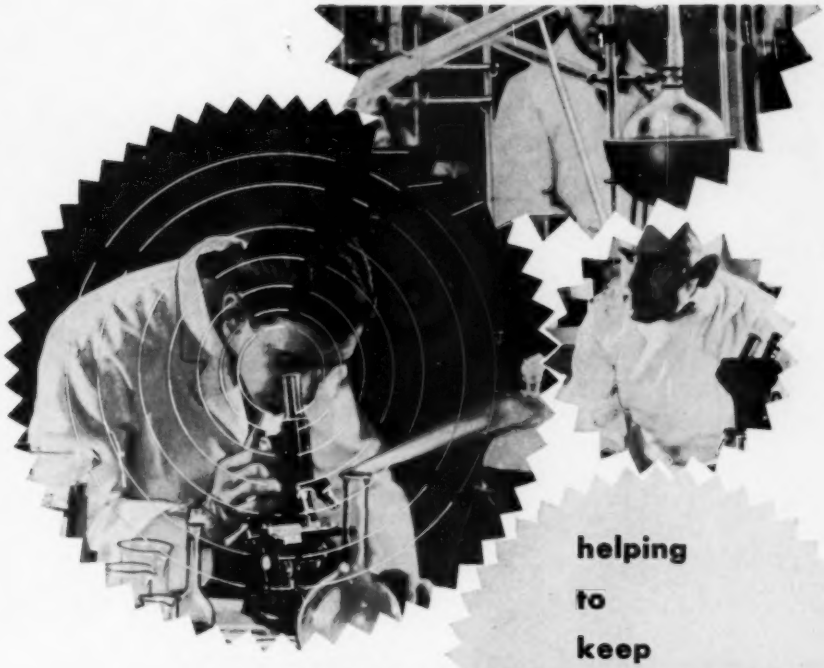
During the period covered by the nomination the nominee shall have been actively engaged in, shall have completed, or shall have published a report upon the line of investigation for which the award is made. During the period of two years prior to the date of nomination, the nominee shall not have been engaged in research under the sponsorship of the Chilean Iodine Educational Bureau, Inc.

The award consists of $1,000 and a diploma setting forth the reasons for selection of the recipient. It may be presented annually at the annual meeting of the ASSOCIATION.

The recipient will deliver a paper or lecture upon the subject of his scientific work at the meeting at which the award is conferred. His paper, or address, will then be published in the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION. In addition to the sum of the award, the recipient will receive an allowance of not more than $250 to defray his expenses in attending the meeting.

The recipient will be selected by an award committee which is appointed by the chairman of the ASSOCIATION'S Council. The present committee includes Justin L. Powers, chairman; Louis Gershenfeld, Charles O. Wilson, Harvey B. Haag, Heber W. Youngken, John C. Krantz, and Frank O. Taylor.

The award is now in its fifth year. The recipient of the first award was Dr. William T. Salter, Yale University pharmacologist; the second recipient was Dr. George M. Curtis, Chairman of the Department of Surgical Research and Professor of Surgery at Ohio State University, and the third was Dr. C. P. Leblond, Professor of Anatomy, McGill University, Montreal, Canada. The fourth award will be shared jointly by Dr. George Moore of the University of Minnesota Medical School and Dr. Moses Ashkenazy, Chief of Neurosurgery, Veterans Hospital, Houston, Tex., and will be conferred during the 1952 Convention of the AMERICAN PHARMACEUTICAL ASSOCIATION.



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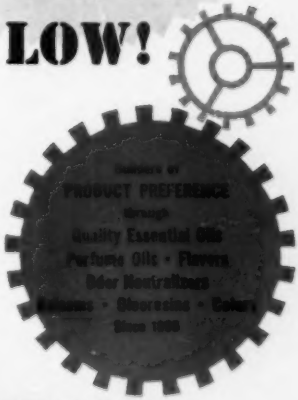

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